Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour

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1. In order to establish the subunit composition of neuronal nicotinic receptors in rat superior cervical ganglia (SCG), their single-channel properties were compared with those of recombinant receptors expressed in *Xenopus* oocytes, using outside-out excised patch recording.

2. The mean main conductance of SCG channels from adult and 1-day-old rats was 34·8 and 36·6 pS, respectively. Less frequent openings to lower conductances occurred both as isolated bursts and as events connected to the main level by direct transitions. There was considerable interpatch variability in the values of the lower conductances.

3. Nicotinic receptors from oocytes expressing $\alpha3\beta4$ and $\alpha4\beta4$ subunits had chord conductances lower than that of SCG neurones (22 pS for $\alpha3\beta4$ and 29 pS for $\alpha4\beta4$).

4. Prolonged recording from both native and recombinant channels was precluded by 'run-down', i.e. channel activity could be elicited for only a few minutes after excision. Nevertheless, SCG channel openings were clearly seen to occur as short bursts (slowest component, 38 ms), whereas recombinant channels opened in very prolonged bursts of activity, the major component being the slowest (480 ms).

5. Addition of the $\alpha5$ subunit to the $\alpha3\beta4$ pair produced channels with a higher conductance than those observed after injection of the pair alone (24·9 *vs.* 22 pS), suggesting incorporation of $\alpha5$ into the channel. Addition of the $\beta2$ subunit did not change $\alpha3\beta4$ single-channel properties. In one out of fourteen $\alpha3\alpha5\beta4$ patches, both ganglion-like, high conductance, short burst openings and recombinant-type, low conductance, slow burst openings were observed.

6. Channels produced by expression in *Xenopus* oocytes of neuronal nicotinic subunits present in rat SCG as a rule differ from native ganglion receptors in single-channel conductance and gross kinetics. While it is possible that an essential nicotinic subunit remains to be cloned, it is perhaps more likely that oocytes either cannot assemble neuronal nicotinic subunits efficiently into channels with the correct composition and stoichiometry, or that they produce post-translational channel modifications which differ from those of mammalian neurones.

Fast synaptic transmission in autonomic ganglia is mediated by the release of acetylcholine and the activation of nicotinic receptors. It has been known for a long time that these receptors differ from those present at vertebrate neuromuscular junctions in their pharmacological properties, especially in their antagonist sensitivity (Paton & Zaimis, 1949). Cloning of the receptor subunits has confirmed these findings. It is known that nicotinic acetylcholine receptors at the neuromuscular junction are pentamers formed by four different subunits ($\alpha1$, $\beta1$, δ and ϵ or γ), none of which is expressed by neurones, but far less is known about neuronal receptors. While neuronal subunits show sequence homology with muscle α subunits, all that is known about how they assemble to form the various types of nicotinic receptors present on neurones is that such receptors are pentamers, which contain two copies of an α subunit (Anand, Conroy, Schoepfer, Whiting & Lindstrom, 1991; Cooper, Couturier & Ballivet, 1991). The numerous neuronal subunits that have been cloned ($\alpha2$–$\alpha9$ and $\beta2$–$\beta4$; see McGehee & Role, 1995) are differentially distributed in different areas of the nervous system, i.e. any given type of neurone expresses only a subset of the possible eleven subunits. For example,

α4 and β2 are more abundant in the central nervous system, and α3 and β4 are more abundant in the periphery, including autonomic ganglia. Furthermore, an indication of the minimum number and type of subunits required to produce a functional receptor comes from heterologous expression in *Xenopus* oocytes: a single α subunit is sufficient in the case of α7–α9, but α2, α3 and α4 can form a functional receptor only if coexpressed with β2 or β4, while α5 and β3 cannot form functional receptors when expressed as a pair with any of the other subunits.

In rat superior cervical ganglia (SCG), mRNA for α3 and β4 is the most abundant, followed by message for α7 and β2; evidence for the presence of α4 and α5 is contradictory (Klimaschewski *et al.* 1994; Mandelzys, Pie, Deneris & Cooper, 1994; Rust, Burgunder, Lauterburg & Cachelin, 1994; Mandelzys, De Koninck & Cooper, 1995; Zoli, Le Novère, Hill & Changeux, 1995). Out of the homomeric or pairwise combinations known to be functional when heterologously expressed (α7, α3β2, α3β4, α4β4 and α4β2), α3β4 is the one that gives the best match of the pharmacological properties of SCG nicotinic receptors at the whole-cell level, followed by α4β4 (Luetje & Patrick, 1991; Covernton, Kojima, Sivilotti, Gibb & Colquhoun, 1994; Wong, Holstad, Mennerick, Hong, Zorumski & Isenberg, 1995).

While α3β4 appears to be a strong candidate for the ganglionic receptor (Wong *et al.* 1995), immunoprecipitation of nicotinic receptors from chick ciliary ganglia (which are reported to contain more α5 message than rat, see Corriveau & Berg, 1993; Mandelzys *et al.* 1994) shows that the α3 and β4 subunits coprecipitate with α5, suggesting that a minimum of three different subunits are necessary to form the ganglion nicotinic receptor (Vernallis, Conroy & Berg, 1993). It is possible that the α5 subunit assembles into functional receptors only when expressed together with another two subunits, since heterologous expression of α4α5β2, α3α5β2 and α3α5β4 receptors has recently been reported (Ramirez-Latorre, Yu, Qu, Perin, Karlin & Role, 1996; Wang *et al.* 1996). The most likely 'triplet' combinations for the sympathetic ganglion are α3α5β4 (Vernallis *et al.* 1993; McGehee & Role, 1995) and α3β2β4 (Papke, 1993; Mandelzys *et al.* 1995).

In the present study we aimed to establish the molecular composition by comparing the single-channel properties of rat recombinant neuronal nicotinic receptors expressed in oocytes with those of adult and neonatal rat SCG neurones, using identical recording conditions for both cell types. We tested both pairwise (α3β4 and α4β4) and triplet (α3α5β4 and α3β2β4) subunit combinations and found clear differences between their conductance and kinetics and those of native SCG receptors. With triplets, we found that the main single-channel conductance of α3β4 was increased by the addition of α5, but not of β2, suggesting that α5 is indeed incorporated into the receptor. Nevertheless, the single-channel properties of most α3α5β4 triplet patches were quite distinct from those of native SCG receptors.

We conclude that, unless the oocyte expression system fails to assemble neuronal nicotinic receptors correctly, the ganglionic nicotinic receptors in the rat do not have the α3β4 structure (with or without β2 or α5).

METHODS

Expression in oocytes

Ovarian lobes were obtained from mature female *Xenopus laevis* anaesthetized by immersion in a 0·2% (w/v) solution (pH 5·6) of ethyl *m*-aminobenzoate (methanesulphonate salt, Tricaine, Sigma) and killed by decapitation followed by destruction of the brain and spinal cord. Ovarian lobes were removed and placed in modified Barth's solution sterilized by filtration (0·22 μm pore filter, Millex-GV, Millipore), with the following composition (mM): NaCl, 88; Tris–HCl, 15; $NaHCO_3$, 2·4; KCl, 1; $MgCl_2$, 0·82; $CaCl_2$, 0·77; with 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin; pH adjusted to 7·4 with NaOH. Clumps of ovarian tissue containing five to ten oocytes each were treated with collagenase (Type IA, Sigma; 245 collagen digestion units per millilitre in Barth's solution, 10–12 oocytes per millilitre) for 75 min at 19 °C and dissected manually into defolliculated oocytes (usually 12–18 h later). Mature (stage V–VI) healthy oocytes were selected for cytoplasmic injection of cRNA (23–69 nl per oocyte; approximate cRNA concentration, 1 μg μl^{-1}) with a fire-polished sharp pipette (12–16 μm tip opening; backfilled with mineral oil, M-5904, Sigma) using a Drummond Nanoject injector. For the synthesis of cRNA, cDNAs coding for the rat nicotinic subunits α3, α4, α5, β2 and β4 (GenBank/EMBL accession codes: α3, X03440; α4, L31620; α5, J05231; β2, L31622; β4, J05232; the clones were kindly provided by Dr Jim Boulter, Salk Institute, La Jolla, CA, USA) were subcloned into pSP64-derived expression vectors (Krieg & Melton, 1984). In these constructs all 5′ untranslated segments from the ACh receptor subunits were replaced by vector-encoded *Xenopus* globin 5′ untranslated segments, with a Kozak consensus sequence ACC**ATG**G (initiating **ATG** – for Met – in bold). Therefore, the residue following Met was mutated in the β2 subunit from Leu to Gly and in the β4 subunit from Arg to Ala. In addition, in the β4 construct silent mutations were introduced in the first twenty-seven nucleotides in order to lower the GC content. Native ACh receptor 3′ untranslated regions were mostly removed and replaced by *Xenopus* globin 3′ untranslated regions (vector-encoded). cRNA was synthesized from linearized plasmid DNA using the RiboMax RNA synthesis kit (Promega, Madison, WI, USA). Reactions were supplemented with 3·75 mM capping nucleotide m7G(5′)ppp(5)G (Pharmacia) in the presence of 1·6 mM GTP. RNA integrity was checked with ethidium bromide-stained denaturating agarose gels. RNA concentration was estimated by comparing intensity of staining.

After injection, oocytes were transferred to multiwell plates (one per well) and incubated in Barth's solution at 18 °C for 3 days before screening for expression by two-electrode voltage clamp. Oocytes which responded to bath application of 30 μM acetylcholine with an inward current of 1 μA or more (at −80 mV) were selected for patch-clamp experiments and transferred to a normal refrigerator (4 °C); they remained viable for at least 1 and often 2 weeks. The vitelline membrane was removed immediately before patch-clamp recording by manual dissection after exposing the oocyte for

5–10 min to hypertonic stripping solution (composition (mM): sodium methylsulphate, 200; KCl, 20; Hepes, 10; $MgCl_2$, 1; pH adjusted to 7·4 with KOH).

Preparation of SCG neurones

SCG from Sprague–Dawley rats (either postnatal day 1 or 'adult', i.e. 4–6 weeks old, killed by decapitation) were 'desheathed' by removing connective tissue in ice-cold rat Ringer solution (see below for composition). The desheathed ganglia were treated at 37 °C for 15 min in 1 mg ml^{-1} trypsin followed by 45 min in 1 mg ml^{-1} collagenase IA plus 1 mg ml^{-1} trypsin inhibitor and 0·1 mg ml^{-1} thermolysin (all from Sigma).

The whole ganglion preparation was then moved to a 1·5 ml recording chamber, where it was held in place by a nylon grid (Edwards, Konnerth, Sakmann & Takahashi, 1989), and continuously superfused with oxygenated Ringer solution at 5 ml min^{-1}. Any satellite cells or extracellular debris were removed from the soma of the neurones by gentle suction using a large cleaning pipette (tip diameter, 3–5 μm). Agonist solutions were applied via the perfusion by manual switching of a Hamilton three-way tap (complete solution exchange was possible within 5 s). All recordings were made within 12 h of dissection.

Electrophysiological recording

Two-electrode voltage clamp and potency ratio analysis for the $\alpha4\beta4$ combination. Experimental design and analysis were as described by Covernton *et al.* (1994). Two-electrode voltage clamp recording was used to obtain current responses to low concentrations of nicotinic agonists from oocytes held at a potential of −80 mV in a calcium-free modified frog Ringer solution (composition (mM): NaCl, 115; Hepes, 10; KCl, 2·8; $MgCl_2$, 2; with atropine, 0·5 μM; pH 7·2). ACh chloride, (−)cytisine, carbachol chloride, (−)nicotine hydrogen tartrate (Sigma) and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP; Aldrich) were bath applied. The aim was to obtain the low concentration limit of the potency ratios relative to ACh, which were defined as the ratio of the concentration of ACh to the equi-effective concentration of the agonist being tested. The potency ratio was determined at the low end of the concentration–response curve, to minimize the effects of desensitization (and thus could differ from those determined from entire curves if the maxima differed for different agonists). In practice, for each agonist two or three points were obtained at the foot of the dose–response curve. Their horizontal distance was determined by non-linear least-squares fitting of Hill equations, constrained to be parallel, as described by Covernton *et al.* (1994).

Single-channel recording. Because our purpose was to compare the single-channel properties of native and recombinant nicotinic receptors, identical experimental conditions were adopted for experiments on the two types of cells. This was particularly important with respect to the external medium and the internal pipette solution, because the conductance and kinetics of nicotinic receptors are strongly affected by the concentration of divalent ions (Mathie, Colquhoun & Cull-Candy, 1991; Papke, 1993; Amador & Dani, 1995).

Single-channel recordings were obtained with an Axopatch-1B amplifier (Axon Instruments) from outside-out patches held at −100 mV. The outside-out configuration was chosen in order to allow us to identify nicotinic channels unequivocally by their activation following agonist application. This was particularly important in oocyte recordings because of the occasional presence of endogenous stretch-activated channels, which have a conductance of 28 pS (Methfessel, Witzemann, Takahashi, Mishina, Numa & Sakmann, 1986). Patches with significant channel activity in control conditions were discarded. Nicotinic channel activity in outside-out patches rapidly declined after excision (see Results); the cell-attached configuration allowed us to obtain longer recordings, which were used for burst analysis of channel behaviour. Cell-attached patches did not allow channel identity to be confirmed by application of acetylcholine; therefore, care was taken to ensure that channels were similar to those in outside-out patches. Patch pipettes were pulled from thick-walled borosilicate glass (1·5 mm o.d., 0·86 mm i.d.; GC150F, Clark Electromedical Instruments), coated with Sylgard® 184 (Dow Corning) and fire-polished before use to a final resistance of 8–10 MΩ for oocytes or 12–18 MΩ for SCG neurones. The internal solution had the following composition (mM): CsCl, 80; CsF, 60; Hepes, 10; EGTA, 10; $CaCl_2$, 1; pH adjusted to 7·2 with KOH. All electrophysiological recording was carried out at room temperature (19–22 °C) in modified rat Ringer solution, with the following composition (mM): NaCl, 150; Hepes, 10; KCl, 2·8; $MgCl_2$, 2; $CaCl_2$, 1; with atropine, 0·5 μM; pH 7·2. Single-channel currents were low-pass filtered at 20 kHz (4-pole Bessel, −3 dB) and stored on FM tape (Store 4, Racal) at a tape speed of 15 inches s^{-1} (bandwidth, DC to 5 kHz, Tchebychef filter).

Analysis

Records were digitized off-line (with a CED-1401plus interface, Cambridge Electronic Design) at 15–25 kHz after filtering at 1·5–2·5 kHz (8-pole Bessel, −3 dB). Single-channel events were analysed with the method of time course fitting with the program SCAN (Colquhoun & Sigworth, 1995), which performs a least-squares fit for both amplitude and duration of events that are long enough. Short gaps and openings were fitted as full closures or as full openings (to the most common amplitude or to the nearest well-defined amplitude), unless there was clear evidence of a transition to a sublevel (i.e. a clearly better match of the fit to the data). Stretches of records with simultaneous openings of more than one channel were excised. A fixed resolution was imposed on the data from each patch after time course fitting and before the analysis of distributions, using as a criterion an expected false event rate lower than 10^{-8} s^{-1}.

Amplitudes. We define an 'open period' as a period during which the channel appeared to be continuously open, regardless of possible transitions between different open levels. Within each open period, changes in the open amplitude were considered as transitions to a different level only if the difference in amplitude was greater than 10% of the amplitude of the full opening. If changes in amplitude of less than 10% had been fitted as transitions in the first stage of analysis, the openings on each side of such transitions were concatenated into a single open period, the amplitude of which was taken as the mean open level, weighted by duration. Only events longer than twice the filter rise time contributed data points to the analysis of amplitudes, which was done by maximum-likelihood fitting of Gaussian distributions to the amplitude distribution histogram. Chord conductance values assume a reversal potential of 0 mV.

Excess open channel noise was measured by fitting Gaussian distributions to separate open point and shut point distributions (see Colquhoun & Sigworth, 1995), and then subtracting the shut point variance from the open point variance.

Durations. Distributions of apparent open periods were displayed as histograms of the distributions of the logarithm of the time interval, using the square-root transformation of the event frequency on the ordinate (Sigworth & Sine, 1987). Distributions

were fitted with mixtures of exponential densities by the maximum-likelihood method. A consistent resolution of 200 μs was imposed on the idealized records obtained from all patches, before obtaining open period distributions. Only patches in which the expected false event rate at this resolution was lower than 10^{-10} s^{-1} were included in the analysis. Patches that had to be heavily excised because of double openings were discarded from the analysis of durations in order to avoid errors introduced by length-biased sampling.

Subconductance sequence analysis. Openings to lower conductance levels were recorded in all patches from adult SCG and in many of these (21 out of 24) they contributed over 10% of the total number of openings. Lower conductance openings were often seen to occur in bursts of activity, distinct from the main conductance openings (Fig. 1). Transition sequence analysis was undertaken in order to investigate whether the lower conductance openings were found in conjunction with the larger conductance openings (therefore representing a substate of the same channel) or in isolation, in which case they may derive from the activity of a separate channel.

For each patch, contiguous amplitude windows were defined on the basis of the means and standard deviations of the Gaussian components used to fit the amplitude distribution. Critical amplitudes were calculated from the amplitude distributions so that equal proportions of high and low amplitude events were misclassified. Transitions in the idealized record were categorized according to the amplitude windows between which they took place. This meant that, in a patch giving a fitted amplitude distribution with two Gaussian components, three contiguous amplitude bands would be defined ((1) shut; (2) lower conductance level; and (3) higher conductance level) and a shutting of the higher conductance level would be classified as a 3–1 transition. Thus, isolated openings to the lower conductance would be represented by a transition sequence 1–2–1, whereas non-isolated events would include all 1–2–3, 3–2–3 and 3–2–1 sequences. In order to include only events whose amplitude could be defined, only those transition sequences in which each of the three sojourns was longer than three times the filter rise time were included in the analysis. As a further precaution, all the original records which were classified as non-isolated events were recalled from the digitized record and visually inspected. Only patches in which more than 1000 transitions remained after imposing the resolution could be used in this analysis. While the majority of SCG patches required a three-component fit to their amplitude distribution, only openings to the highest and the intermediate component were numerous enough to be used for subconductance sequence analysis.

RESULTS

The single-channel records in Fig. 1 show the activity evoked by the nicotinic agonist cytisine (1 μM) in outside-out patches from adult rat SCG, and from *Xenopus* oocytes injected with RNA for the $\alpha 3\beta 4$ subunit combination.

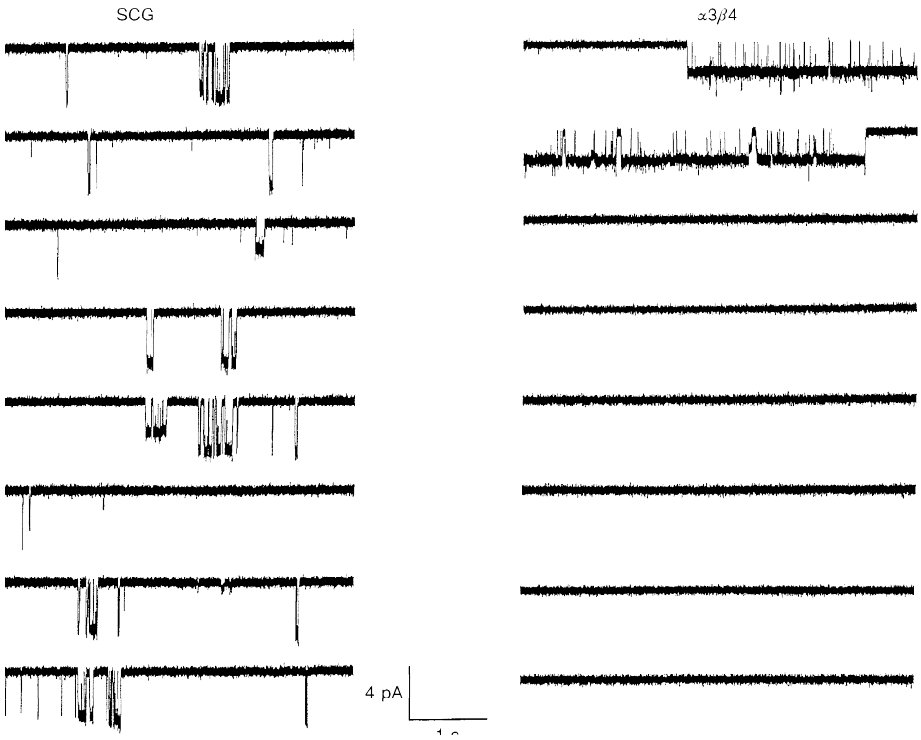

Figure 1. Single-channel activity elicited by 1 μM cytisine in outside-out patches from an adult SCG neurone and an oocyte expressing the $\alpha 3\beta 4$ subunit combination

The traces are continuous records, in both cases obtained approximately 2 min after patch excision. No more channel openings could be observed in the oocyte patch after the burst shown here. Holding potential, −100 mV; filtered at 1·5 kHz.

These results illustrate the profound differences between the behaviour of native and recombinant channels that were observed throughout this study. First, most channel openings in patches from SCG were to a conductance level of approximatively 35 pS (in good agreement with Mathie *et al.* 1991). All the recombinant receptors tested had lower conductance levels than this, as illustrated by the $\alpha 3\beta 4$ combination in Fig. 1, which had a conductance of 21 pS. Second, patches from SCG neurones also displayed openings to smaller conductance levels, which were less frequent and often occurred in isolation, in separate bursts. Third, openings by SCG channels occurred in short bursts (of the duration expected from the synaptic current decay; see Mathie *et al.* 1991), whereas in the majority of recombinant patches openings were clustered in very prolonged bursts of activity.

SCG and recombinant nicotinic channels had in common the tendency to 'run down' rapidly after the patch was excised. Channel activity could be elicited only for a short time, usually less than 5 min, and the run-down was independent of agonist exposure.

Single-channel amplitudes

The amplitudes of channel openings in SCG neurones are unusually variable (compared with muscle nicotinic or NMDA receptors), as already noted by Mathie *et al.* (1991), and the same is true of recombinant neuronal nicotinic receptors. Because of the usefulness of channel conductances for receptor classification in other systems (e.g. Mishina *et al.* 1986; Stern, Béhé, Schoepfer & Colquhoun, 1992; Wyllie, Béhé, Nassar, Schoepfer & Colquhoun, 1996), we shall next describe the channel amplitudes in detail.

SCG neurones from adult rats

The mean level of the most prevalent nicotinic conductance in SCG neurones (i.e. one value for each patch) was $34{\cdot}8 \pm 0{\cdot}9$ pS ($n = 23$), with a mean area of $70{\cdot}9 \pm 3{\cdot}5\%$. This is similar to the value reported by Mathie *et al.* (1991). Examples of channel openings are shown in Fig. 2*A*.

The consistency of this value across patches, however, belies the complexity of the underlying conductance pattern: three components were necessary in order to fit the distribution of single-channel currents satisfactorily in fourteen patches out of twenty-three (Fig. 2*B* and *C*). Nevertheless, interpatch

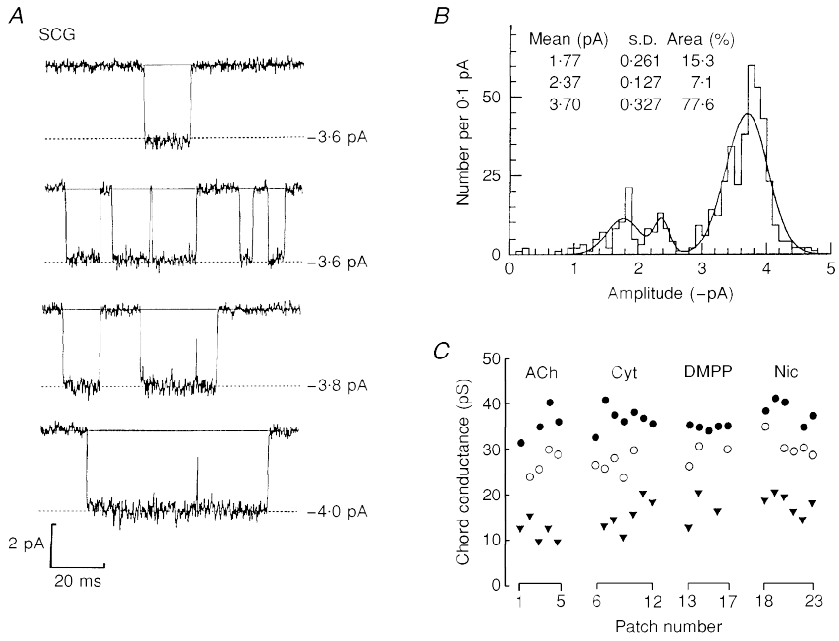

Figure 2. Conductance properties of nicotinic receptors from adult SCG

A, examples of single-channel openings to the main conductance level of SCG. Note the variation in open channel amplitude within each opening. Records are from an outside-out patch held at −100 mV (filter, 1·5 kHz; the patch is the same as the one shown in Fig. 1). *B*, distribution of single-channel current amplitudes obtained by time course fitting of the patch in Fig. 1. Note the three Gaussian components needed to fit the data and the large standard deviation of the Gaussian, particularly for the main 3·7 pA component. *C*, single-channel conductances in adult SCG patches. All the conductance components fitted to the amplitude distribution of each patch are shown plotted against patch number. Note that most patches required three components (●, ○ and ▼; as in *B*) and that choice of agonist did not affect conductances. Cyt, cytisine; Nic, nicotine.

variation was such that the conductance values obtained in this manner did not fall into three clear-cut bands when the results from all the patches were collated (Fig. 2*C*). The largest component averaged 36·3 ± 0·6 pS ($n = 22$; range, 30–41 pS), and this component was usually predominant. The second component overlapped too much with the first to be detected as a clear band, but the smallest component was relatively well separated (mean, 15·3 ± 0·8 pS; range, 10–21 pS; $n = 20$ in 23 patches). The relative abundance of each of the three components, as judged from the area of each fitted Gaussian, was also quite variable. The largest component was detected in twenty-two out of twenty-three patches and represented 68·4 ± 4·3% of the openings (range, 29·6–100%), The second largest component (19 out of 23 patches) constituted 29·2 ± 5·8% of the openings (range, 3·5–97·6%), whereas only a minority of the openings fell into the smallest conductance component (17·4 ± 2·8%; range, 2·4–44·1%; 19 out of 23 patches).

Figure 3 shows the results of subconductance sequence analysis (see Methods). Data from twenty-one patches were pooled in order to compare how often the lower conductance appeared in isolation or in conjunction with a higher conductance opening. The majority of sojourns in either the lower or the higher conductance bands occurred as isolated events (86 and 95%, respectively; ▨ in Fig. 3*B*); nevertheless, a small number of events in which a sojourn started or ended with a direct transition to a different conductance level were also observed (■ in Fig. 3*B*). Therefore, the same channel which gives rise to the higher, or main conductance can open to lower levels.

SCG neurones from neonatal rats

A subtly different picture was observed in patches from SCG neurones of postnatal day 1 rats. With respect to the main conductance in the patches, there were clearly two populations within our sample (see Fig. 7): the majority (16 out of 20 patches) had a large main conductance, similar to adult neurones (36·6 ± 0·6 pS; mean area, 71·5 ± 4·7%), but in four patches a smaller conductance predominated (19·4 ± 0·6 pS; mean area, 72·1 ± 11·4%)

Heterogeneity of SCG nicotinic channels

One hypothesis consistent with these observations is that many (but not all, see the results of subconductance sequence analysis) of the smaller openings in SCG are produced by a distinct population of receptors, possibly made up of different subunits from the main larger conductance channels. This hypothesis can be tested, at least in its simplest form, because 'pair' recombinant receptors of different composition have different sensitivity to agonists. In particular, cytisine is a potent agonist on β4-containing receptors and a poor

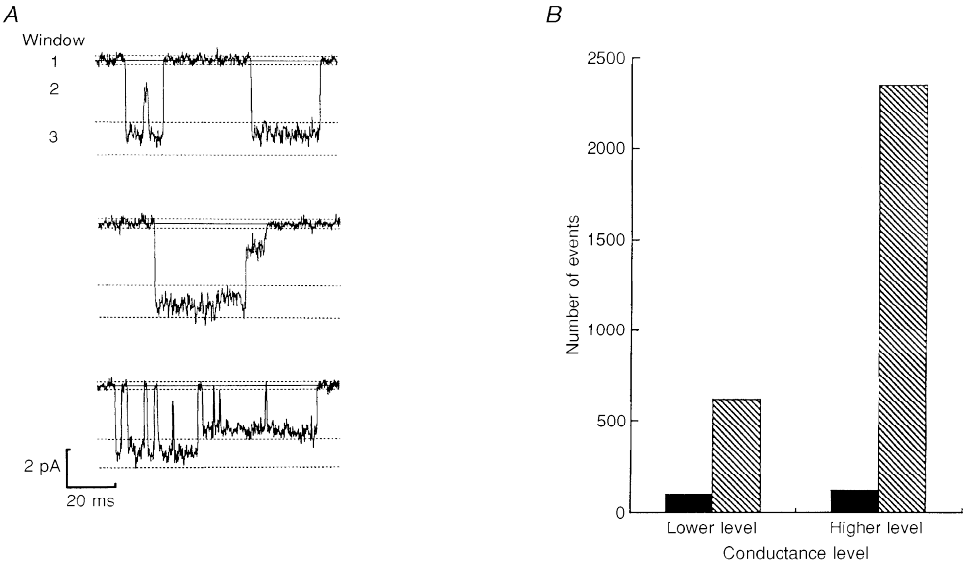

Figure 3. Sequence analysis of transitions from SCG channels

This was done to investigate what proportion of openings are connected by direct transitions to events of different amplitudes and are therefore likely to arise from a single type of channel molecule. *A*, channel openings from an adult SCG outside-out patch. The dotted lines show the three contiguous windows defined in this patch in order to classify sojourns as shut (1) or as lower and higher conductance (2 and 3; note that the latter is the main level in the majority of patches). Examples are shown of both isolated and non-isolated openings. *B*, the histogram shows the number of isolated events (▨) relative to non-isolated events (■) for both the lower (i.e. window 2) and higher (i.e. window 3) conductance openings. Data pooled from 21 patches.

one on $\alpha3\beta2$, whereas DMPP is potent on $\alpha3\beta2$ and poor on $\alpha3\beta4$ (Luetje & Patrick, 1991; Covernton *et al.* 1994). If the conductance heterogeneity in SCG patches were due to the presence of different populations of 'pair' receptors and if each of these can be assumed to have one relatively homogeneous conductance (but see below), we would expect different agonists to evoke different conductances. However, no such difference was observed when we compared openings evoked by low concentrations (1–3 μM) of ACh, cytisine, DMPP or nicotine (Fig. 2*C*). Similar results were obtained in patches from newborn rat SCG neurones. The implication is that all of the conductances in SCG derive from a single type of receptor molecule. Alternatively, different receptor populations may exist, each capable of opening to several conductance levels and endowed with a broadly similar sensitivity to agonists.

Amplitudes of pairwise recombinant combinations: $\alpha3\beta4$ and $\alpha4\beta4$

The mean amplitude of the most prevalent conductance level observed in patches from oocytes injected with $\alpha3\beta4$ was 22·0 ± 0·72 pS (range, 19–31 pS; $n = 14$; mean area, 82·3 ± 4·8 %), and with $\alpha4\beta4$ it was 29·0 ± 1·24 pS (range, 23–35 pS; $n = 10$; mean area, 64·9 ± 8·2 %; see Fig. 4*A* and *C*). More than one distinct conductance was usually present in each patch. As shown in Figs 4*B* and 5, the amplitude distribution could be fitted satisfactorily with one component only in four out of fourteen $\alpha3\beta4$ patches. As with SCG channels, the conductance values from each patch could not be grouped easily into clearly defined classes.

Sublevels in recombinant channels

As in SCG cells, secondary levels not connected to the main conductance by direct transition were observed (particularly in cell-attached patches, which lasted longer) and again, as in SCG patches, the different conductance levels mostly occurred in separate bursts (data not shown). In addition, direct transitions could be observed between the main conductance and some of the secondary conductances of recombinant receptors. Examples of this behaviour are detectable in Figs 1, 4*A* and *C* and 6. However, the amplitude and frequency of sublevels was not sufficiently consistent to make their presence a useful diagnostic criterion for subunit composition.

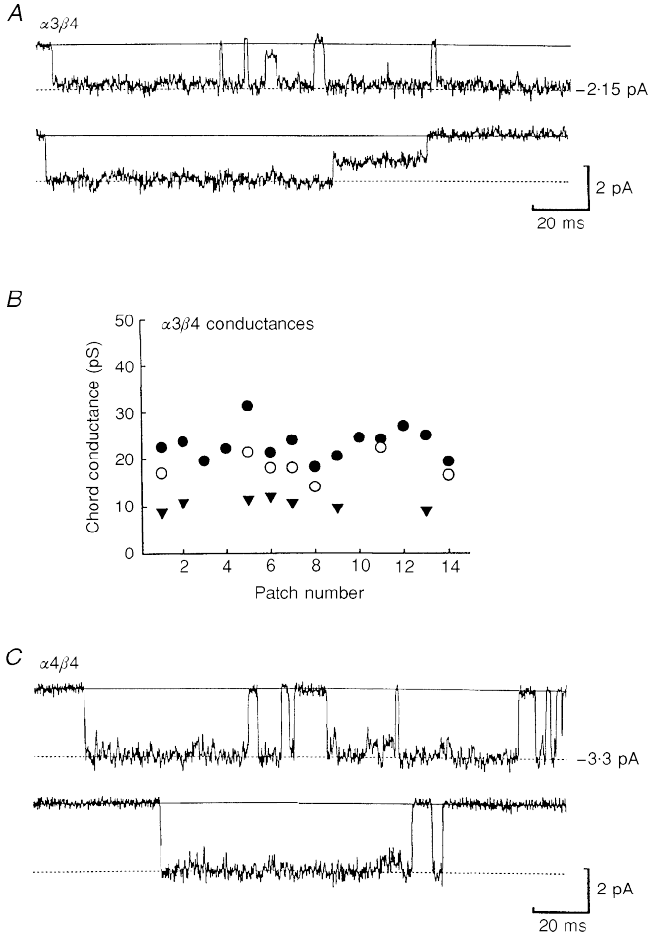

Figure 4. Conductance of recombinant 'pair' combinations: $\alpha3\beta4$ and $\alpha4\beta4$

A, examples of single-channel openings to the main conductance of $\alpha3\beta4$ channels recorded from outside-out patches from *Xenopus* oocytes. Holding potential, −100 mV; filter, 1·5 kHz. Note the long apparent open times and the clear direct transitions to conductance sublevels. *B*, single-channel conductances in oocyte-expressed $\alpha3\beta4$ patches. All the conductance components (indicated by ●, ○ and ▼) fitted to the amplitude distribution of each patch are shown plotted against patch number. *C*, examples of single-channel openings to the main conductance of $\alpha4\beta4$ channels recorded from outside-out patches from *Xenopus* oocytes. Holding potential, −100 mV; filter, 1·5 kHz.

Table 1. Potency ratios for nicotinic agonists in oocytes and rat SCG neurones

	Cytisine	DMPP	Nicotine	Carbachol
$\alpha4\beta4$	30·30 ± 9·18	0·045 ± 0·020	0·952 ± 0·662	0·091 ± 0·048
SCG*	4·76 ± 0·70	2·50 ± 0·24	1·98 ± 0·13	0·361 ± 0·022
$\alpha3\beta2$*	0·020 ± 0·012	1·62 ± 0·49	0·038 ± 0·012	0·068 ± 0·028
$\alpha3\beta4$*	5·06 ± 0·76	0·43 ± 0·08	1·10 ± 0·31	0·25 ± 0·04

* Potency ratios for SCG, $\alpha3\beta2$ and $\alpha3\beta4$ are adapted from Covernton *et al.* (1994). $n = 6$–7 for $\alpha4\beta4$, 3–4 for SCG, 7–21 for $\alpha3\beta2$ and 4–7 for $\alpha3\beta4$. Values are means (± approximate S.D.) from Fieller's theorem (Colquhoun, 1971) and are concentration ratios relative to ACh. A value of 30·3 for cytisine means that cytisine is 30·3-fold more potent than ACh (see Methods).

Agonist potency ratios: the $\alpha4\beta4$ combination

Because $\alpha4\beta4$ channels had the highest main conductance value of all the recombinant combinations tested and were thus closest to the values seen with SCG channels, it was important to measure the sensitivity of this combination to different nicotinic agonists, in order to compare it with that of SCG channels. The rank order of agonist potency for $\alpha4\beta4$ (cytisine $\gg$ ACh $\approx$ nicotine $\gg$ carbachol $\approx$ DMPP) was found to be a poorer match to SCG than that of the $\alpha3\beta4$ combination (Covernton *et al.* 1994; Table 1).

Amplitudes of 'triplet' combinations: $\alpha3\beta2\beta4$ and $\alpha3\alpha5\beta4$

Addition of the $\alpha5$ subunit to the $\alpha3\beta4$ pair produced a significant increase in the most common conductance level in outside-out patches to 24·9 ± 0·6 pS ($n = 13$; $P = 0·0055$, two-tailed randomization test, Colquhoun, 1971; mean area, 82·0 ± 5·2 %). The amplitude distribution of $\alpha3\alpha5\beta4$ patches was usually fitted with two components (Fig. 6).

Unlike $\alpha5$, which cannot form functional $\alpha5\beta4$ or $\alpha3\alpha5$ receptors, $\beta2$ can form an additional receptor type, $\alpha3\beta2$; it

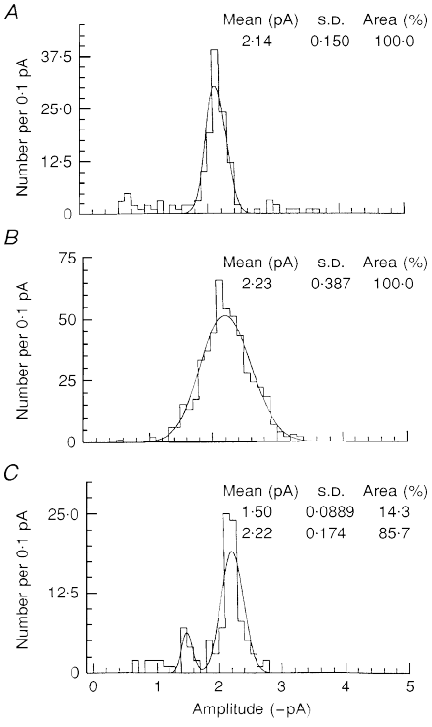

Figure 5. Examples of distributions of single-channel current amplitudes obtained by time course fitting of $\alpha3\beta4$ patches

Note that although the means of the main Gaussian components are similar, their standard deviations are markedly different. Furthermore, the number of components necessary for the optimal fit of the amplitude distribution was variable.

is therefore possible that the injection of $\alpha3\beta2\beta4$ may result in at least three types of receptors, the two pairs $\alpha3\beta2$ and $\alpha3\beta4$ and the triplet $\alpha3\beta2\beta4$. In order to reduce this potential problem, we chose to use low concentrations of cytisine as the agonist for this subunit combination, the rationale being that, if such a triplet receptor were ganglionic, it would have to be highly sensitive to cytisine. This agonist could therefore be expected to activate only two populations, $\alpha3\beta4$ and, if it is ganglion-like, the triplet $\alpha3\beta2\beta4$.

We found that the most common conductance elicited by cytisine in patches from oocytes injected with $\alpha3\beta2\beta4$ was $22{\cdot}1 \pm 0{\cdot}5$ pS ($n = 7$; area, $69{\cdot}8 \pm 8{\cdot}5$%). This value is essentially the same as that observed in $\alpha3\beta4$ patches, suggesting that either $\beta2$ is not incorporated into a 'triplet' receptor, or that it does not affect its conductance. Alternatively, 'triplet' $\alpha3\beta2\beta4$ receptors may not be sensitive to cytisine, which would exclude the combination as a possible candidate for the ganglion receptor. A minimum of two conductance levels were required to fit amplitude distributions adequately.

Amplitudes: a summary

Figure 7 summarizes the results outlined so far, by showing in *A* the most common conductance value in each patch. It is clear that nicotinic receptors from both adult and neonatal SCG open to a main conductance level which is larger than any of those observed with recombinant combinations in oocytes. Indeed, the combination that most closely approaches the high native conductances is $\alpha4\beta4$, which is quite different from SCG receptors in its lack of sensitivity to DMPP.

A more complex picture appears if we include all the conductance levels which represent 10% or more of the opening in the patches (Fig. 7*B*). The most striking effect of this inclusion is that conductances lower than the main level of 35 pS become apparent for SCG channels. These smaller conductances, which can occur in separate bursts, fall into a range similar to that of recombinant channels.

Because these lower conductance openings can also occur directly linked to the main conductance and because their agonist sensitivity is similar to that of the main conductance, it is unlikely that these openings are produced by receptors similar to pair combinations or to the triplet $\alpha3\alpha5\beta4$. Further evidence was obtained by comparing the kinetics of recombinant and native channels.

As described above, the run-down phenomenon severely limited the number of openings that could be recorded from outside-out patches. As a consequence, the only kinetic property that could be examined in most patches was the

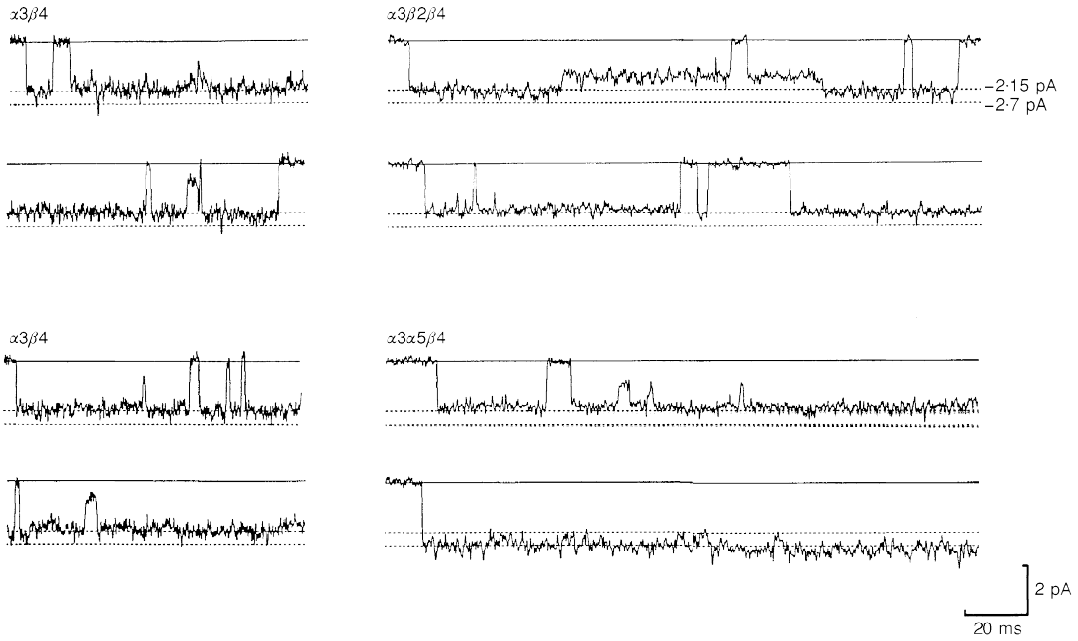

Figure 6. Examples of single-channel currents from oocyte-expressed $\alpha3\beta2\beta4$ and $\alpha3\alpha5\beta4$ 'triplet' subunit combinations

The openings shown are to the main conductances of each combination (holding potential, −100 mV; filtering, 1·5 kHz). Note that addition of $\alpha5$ to the $\alpha3\beta4$ combination results in the appearance of a larger conductance level (27 pS; lower trace), mixed with the $\alpha3\beta4$-like 21·5 pS openings (upper trace). No change in the $\alpha3\beta4$ conductances was produced by the addition of $\beta2$. Neither $\beta2$ nor $\alpha5$ changed the long apparent open times characteristic of recombinant $\alpha3\beta4$ channels.

Table 2. Open period distribution: SCG *vs.* recombinant receptors

	τ_1 (ms)	Area_1 (%)	n	τ_2 (ms)	Area_2 (%)	n
SCG	20·2 ± 2·4	55·9 ± 7·5	7/7	1·11 ± 0·24	44·1 ± 7·5	7/7
$\alpha3\beta4$	47·8 ± 9·1	81·4 ± 9·9	7/12	2·90 ± 0·60	55·0 ± 11·0	8/12
$\alpha4\beta4$	33·4 ± 6·0	62·7 ± 12·5	7/10	2·47 ± 0·95	55·0 ± 13·1	9/10
$\alpha3\beta2\beta4$	35·7 ± 5·5	61·0 ± 14·0	6/6	3·07 ± 0·90	59·0 ± 11·0	4/6
$\alpha3\alpha5\beta4$	31·6 ± 7·0	69·9 ± 12·3	5/7	2·99 ± 0·32	79·2 ± 20·8	2/7

Columns headed τ and Area give the average (± S.D.) of the values of the means and of the relative areas of the components fitted to the open period distribution. n values are the number of patches with a component divided by the total number of patches.

distribution of apparent open periods. The results of this analysis (Table 2) show that apparent open period distributions from recombinant channels could usually be fitted with a main slow component (time constant, $\tau_1 = 30$–48 ms), alone or with a shorter one ($\tau_2 \approx 3$ ms). In a minority of recombinant patches, different behaviour was observed, and only short apparent open periods were detected, though the amplitudes in these patches were similar to the others.

The apparent open period distribution for SCG channels had two components with similar areas, the slower of which was consistently faster than the slowest component observed in recombinant patches ($\tau_1 = 20$ ms for SCG compared with 30–48 ms for recombinants). The faster component for SCG neurones was consistently faster ($\tau_2 = 1$–1·5 ms) than the fast component for recombinant receptors ($\tau_2 = 2{\cdot}5$–3 ms). Analysis of low amplitude openings alone showed that their lengths were similar to the overall distribution, although the number of transitions that could be analysed was small. This suggests that, regardless of the conductance level, the open channel behaviour of native SCG channels is different from that of recombinant receptors.

In eight patches from recombinant channels enough openings were recorded to allow a shut time distribution to be fitted, so a suitable critical gap length (Colquhoun & Sigworth, 1995) could be chosen for analysis of burst lengths. In these patches (4 with $\alpha3\beta4$, 2 with $\alpha3\beta4\beta2$ and 2 with $\alpha3\alpha5\beta4$) the slowest component (which was the main component in 7 out of 8 patches) had an average τ of 480 ± 11 ms (range, 193–920 ms). The slow burst length component for SCG channels was 31·8 ± 4·0 ms ($n = 23$); this represented only 23·3 ± 3·5% of the bursts, the main component being the shortest (0·28 ± 0·08 ms; area, 54·8 ± 4·9%).

Ganglion-like behaviour in one $\alpha3\alpha5\beta4$ patch

One out of the fourteen $\alpha3\alpha5\beta4$ patches was quite different from all the others in that it showed channels that were clearly very similar to those seen in SCG cells, albeit mixed with the usual recombinant type of channel opening. Figure 8 shows examples of the two sorts of event seen in

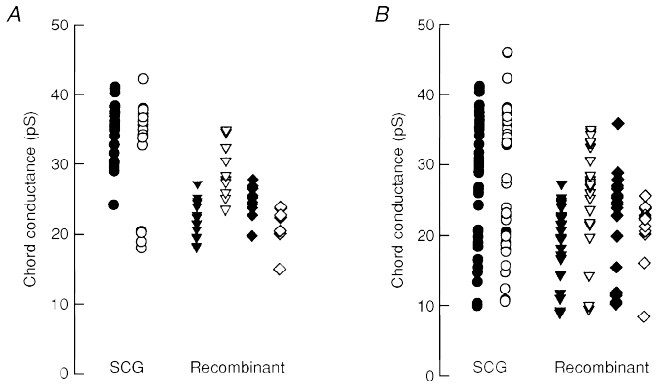

Figure 7. Summary of the conductance levels observed in native and recombinant nicotinic receptors

A, main conductance levels (e.g. for each patch the component which represented the majority of the openings). *B*, all the conductance levels representing more than 10% of the openings in a patch. ●, adult SCG; ○, postnatal day 1 SCG; ▼, $\alpha3\beta4$; ▽, $\alpha4\beta4$; ◆, $\alpha3\alpha5\beta4$; ◇, $\alpha3\beta2\beta4$.

this patch. The openings shown on the left have a conductance of 34·3 pS, and they occur in short bursts (apparent open periods, 3·6 ms on average), just like those in ganglion cells. The openings on the right have a conductance of 26·1 pS, with direct sublevel transitions, and are clustered in long bursts with average apparent open period duration of 41 ms, very much like those in other $\alpha 3\alpha 5\beta 4$ patches.

Open channel noise: native *vs.* recombinant channels

Our data agree with those of Mathie *et al.* (1991), which showed that ganglion nicotinic channels openings are noisy, possibly because of open–open transitions to discrete, but very similar conductance levels. A quantification of this phenomenon (which is also somewhat inconsistent, e.g. it can be absent from a proportion of the openings) can be provided by the *excess* open channel noise. Expressed as a coefficient of variation (see Methods), this measures the extent to which the current is noisier when the channel is open than when it is shut and was found to be $10{\cdot}0 \pm 0{\cdot}5\%$ of the main conductance level of adult SCG patches ($n = 23$). This is much greater than for a muscle-type receptor, which has little excess open channel noise. The excess noise is predominantly low frequency and does not resemble unresolved flickering (Mathie *et al.* 1991). It was interesting to observe that recombinant neuronal nicotinic channels *did* resemble native receptors in this respect: the excess open channel noise for the main conductance component was $12{\cdot}2 \pm 1{\cdot}5\%$ for $\alpha 3\beta 4$ ($n = 14$), $8{\cdot}1 \pm 1{\cdot}2\%$ for $\alpha 4\beta 4$ ($n = 10$), $10{\cdot}0 \pm 0{\cdot}5\%$ for $\alpha 3\beta 2\beta 4$ ($n = 6$) and $11{\cdot}7 \pm 1{\cdot}3\%$ for $\alpha 3\alpha 5\beta 4$ ($n = 12$).

DISCUSSION

Our data clearly show that expression in *Xenopus* oocytes of the nicotinic subunits present in rat sympathetic ganglia produces channels that do not resemble those present in native SCG neurones, in either conductance or kinetics. This could be either because the molecular composition of the native receptor is different from the recombinant combinations we tested or because the oocyte expression system fails to produce neuronal nicotinic receptors accurately and/or efficiently.

How can the subunit composition of receptors be determined?

The aim of our work was to identify the subunit composition of the nicotinic receptor in mammalian sympathetic ganglia by comparing recombinant combinations and native receptors. This problem is the modern, molecular form taken by one of the crucial questions of classical pharmacology, namely the characterization and comparison of receptors present in different tissues or cell types. The methods (based on the functional properties of receptors) that can be used to assess subunit composition include the following.

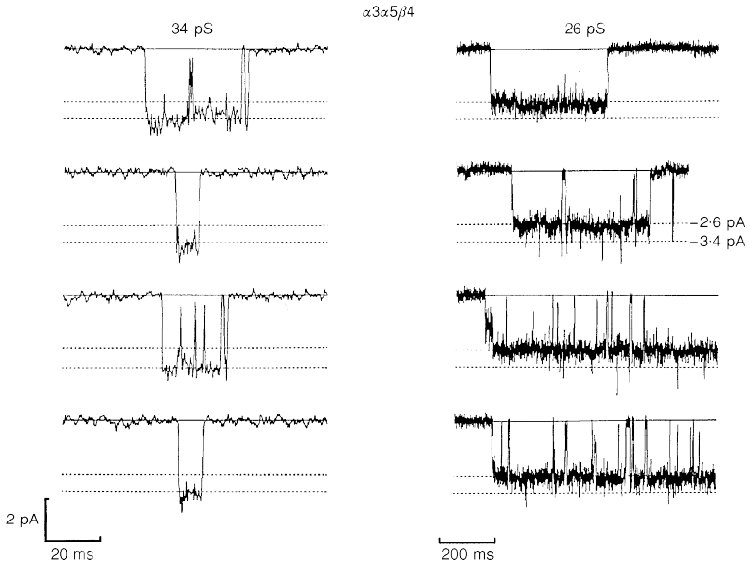

Figure 8. Ganglion-like channels in one $\alpha 3\alpha 5\beta 4$ patch

Example of ganglion-like channel activity in one patch from an oocyte expressing the $\alpha 3\alpha 5\beta 4$ combination (left). Note that the 34 pS openings occur, like those of SCG receptors, in short bursts. In the same patch, channel openings that are more typical of the 'triplet' recombinant combination were also observed (right); these had a main conductance of 26 pS and occurred in long clusters of openings.

Macroscopic methods. At the macroscopic level, in our case that of whole-cell responses, the most robust technique involves using the Schild method (Arunlakshana & Schild, 1959) to obtain the dissociation constants for a competitive antagonist in the two tissues being examined; identity of the dissociation constants for a range of antagonists provides evidence for the identity of the two receptor types. This option is precluded for neuronal nicotinic receptors by the fact that most known antagonists are not competitive, and by the lack of antagonists that distinguish between different subunit combinations.

An alternative approach exploits the possible differences in the agonist sensitivity of the different receptor subtypes. A drawback of this method is that a simple measurement, say the EC_{50} of an agonist, does not allow estimation of a physical constant (comparable with the dissociation constant for an antagonist) which is characteristic of the receptor and can be compared robustly across tissues. An additional problem is that the value obtained for the EC_{50} is critically dependent on having a precise estimate of the maximum response, and full concentration–response curves are often difficult to measure accurately, particularly near the maximum response, because of desensitization and/or channel block. Furthermore, the extent of the error that results from desensitisation is likely to be different for small cells (like sympathetic neurones) and big cells (like oocytes), because the latter do not permit fast application of the agonist. It is for this reason that here, as in the study of Covernton *et al.* (1994), we have measured the low concentration limit of the potency ratio (see the section of the Methods on two-electrode voltage clamp), which does not require knowledge of the maximum response and so is likely to be a more reproducible measure of relative potency than EC_{50} estimates. Note, though, that these two ways of estimating relative potency will not, in principle, give the same results if the agonists being compared have (genuinely or artifactually) different maximum responses. Problems of this sort are probably one reason for the wide range of values that have been reported for the EC_{50} of ACh on oocytes expressing rat $\alpha 3\beta 4$ subunits (63–219 μM in 0 mM Ca^{2+}; Cohen, Figl, Quick, Labarca, Davidson & Lester, 1995; Zwart, Oortgiesen & Vijverberg, 1995).

Single-channel methods. Since the subunit composition of the receptor determines the nature of the amino acid residues lining the channel pore, the physical properties of the channel (conductance, selective permeability, channel block and rectification) may prove diagnostic of a particular molecular structure. However, determining the ionic permeability and current rectification of channels expressed in oocytes requires prolonged recording in excised patch configurations (to allow control of the internal ionic composition); because of the 'run-down' phenomenon, this is not feasible for neuronal nicotinic receptors. This leaves the possibility of measuring single-channel conductance, which can be done even if only relatively short recordings can be obtained, the only essential precaution being to use identical ionic media for native and recombinant receptors. Single-channel conductance ought to be an indication of the identity of a channel assembly which is robust to tissue differences since, to our knowledge, only one study has shown effects of post-translational modifications of receptors on conductance (van Hooft & Vijverberg, 1995).

Single-channel conductance has proved a very useful tool in the molecular characterization of channels. The existence of a developmental switch between ϵ and γ subunit in the nicotinic receptor at neuromuscular junction was confirmed by measuring single-channel conductance of recombinant γ- and ϵ-containing combinations in oocytes (Mishina *et al.* 1986). By the same token, the main and secondary single-channel conductances of native NMDA receptors from different areas of the CNS show a good agreement with those of oocyte-expressed recombinant NMDA channels containing the NR2 subunit type which is most abundant in each area (Stern *et al.* 1992; Wyllie *et al.* 1996). NMDA receptors, when expressed in mammalian cell lines, also showed very similar conductance properties to those seen in oocytes or native receptors (Stern, Cik, Colquhoun & Stephenson, 1994), so these properties are robust in this case.

For neuronal nicotinic receptors, the main problem in comparing native and recombinant conductances stems from the profound variability of conductance values observed in the present study for both native and recombinant channels (Mathie *et al.* 1991). Despite the complication added by the variability of conductances, our data show that the largest conductances observed in SCG are consistently larger than those of any of the recombinant combinations tested. This poses a problem which extends beyond the SCG: other preparations (most notably adrenal medulla, parasympathetic ganglia and PC12 cells) express a set of nicotinic subunits similar to that of sympathetic ganglia, but have nicotinic receptors with large main conductance levels (for a review see Papke, 1993).

Receptor kinetics. Another set of physical properties that can be used to characterize a ligand-gated ion channel is its behaviour in response to agonist application, in other words the kinetics of activation–deactivation and desensitization (and their voltage dependence) and the frequency of different sublevel transitions. The methods necessary to study these properties require either prolonged recording of steady-state channel activity or fast concentration or voltage jumps. For oocyte-expressed channels, all of these are best done in excised patches and are not feasible for neuronal nicotinic receptors because of the speed of receptor 'run-down'. In the present study the patches that lasted longest did provide us with some steady-state data, which reinforced the conclusion drawn from the conductance analysis that recombinant nicotinic receptors differ from native receptors.

Choice of subunit combinations to be tested

The combinations to be tested (out of the many functional) were selected first of all on the basis of presence of the

mRNA for the different subunits and relative mRNA abundance. This, however, may or may not reflect the relative abundance of subunit *protein* on the somatic membrane of the neurone; furthermore, it is controversial whether or not mRNA for the α4 and α5 subunits is present at all in the *rat* ganglion (Klimaschewski *et al.* 1994; Mandelzys *et al.* 1994; Rust *et al.* 1994; Zoli *et al.* 1995).

Other indications were provided by the pharmacological properties of ganglionic and recombinant receptors. For instance, while mRNA for the α7 subunit is present in SCG, the α7 blocker α-bungarotoxin does not reduce the inward current or the rise in intracellular calcium ions that are evoked by fast agonist application (Trouslard, Marsh & Brown, 1993; Mandelzys *et al.* 1995). The relative potency of cytisine, nicotine and ACh in ganglia resembled strongly the profile of $\alpha3\beta4$ receptors, although the potency of DMPP may indicate a β2-containing combination (see Table 1 and Covernton *et al.* 1994; Mandelzys *et al.* 1995). These data have been taken as an indication of a likely $\alpha3\beta2\beta4$ 'triplet' composition for the ganglion receptor (Papke, 1993; Mandelzys *et al.* 1995). The α7 subunit is unlikely to contribute to an α3-containing receptor, since in chick ciliary neurones receptors containing α7 appear to be separate from the 'synaptic' $\alpha3\alpha5\beta4$ receptor population (Conroy & Berg, 1995).

Furthermore, antisense experiments in chick lumbar sympathetic neurones suggested that α3, rather than α4 or α7, is the main contributor to ganglion receptors (Listerud, Brussaard, Devay, Colman & Role, 1991). However, extrapolation from data obtained in chick neurones has to be done with caution because there may be substantial differences between rat and chick ganglia, which show differences in the subunits they express and in their changes during development (Listerud *et al.* 1991; Mandelzys *et al.* 1994; Rust *et al.* 1994).

Why do recombinant receptors not resemble the native ganglion channels?

The first and simplest explanation would be that one (or more) nicotinic subunits, essential to ganglion receptors, has yet to be cloned. In order for this subunit to have escaped detection until now, it would have to be considerably different from the known ones, but this is not impossible, because all the neuronal subunits cloned so far were found because of their sequence homology with the muscle α subunit. It is therefore conceivable that neuronal subunits more similar to the muscle non-α clones await discovery. Pugh, Corriveau, Conroy & Berg (1995) have shown that chick sympathetic ganglion receptors do contain a protein which is distinct from all the known subunits. However, this protein coprecipitates with the subpopulation of receptors which bind α-bungarotoxin and contain α7, which differ from the major rat ganglion receptor. Injecting oocytes with native mRNA isolated from rat SCG (or sympathetic chain ganglia) and characterizing at single-channel level the nicotinic receptors thus expressed is a possible approach to testing the hypothesis that a hitherto uncloned subunit contributes to the properties of ganglion nicotinic receptors.

The alternative explanation is that the oocyte somehow fails to assemble the 'right' receptor even if it is given the right subset of subunits. One possibility is that receptors expressed in oocytes may be subject to post-translational modifications different from those carried out by neurones. Oocytes glycosylate *Torpedo* electric organ nicotinic receptors differently from *Torpedo* (Buller & White, 1990) and have a notoriously high basal level of protein kinase A activity (Hoffman, Ravindran & Huganir, 1994). Nevertheless, these differences do not seem to matter too much for the expression of NMDA receptors, for which there is close agreement between the properties of native receptors and those of recombinant receptors, regardless of the expression system (Stern *et al.* 1994).

For muscle-type nicotinic receptors, the situation is not quite so clear, but it may well be that the oocyte is not a totally reliable expression system for muscle nicotinic receptors either (see Gibb, Kojima, Carr & Colquhoun, 1990; Edmonds, Gibb & Colquhoun, 1995). Problems with expression of muscle-type nicotinic receptors in oocytes apparently involve channel kinetics (sometimes in a fairly subtle fashion; see Gibb *et al.* 1990) to a greater extent than the main single-channel conductance. Although channel conductances for muscle nicotinic receptors expressed in oocytes may certainly be more heterogeneous than is seen for native receptors in adult endplates (see Kullberg, Owens, Camacho, Mandel & Brehm, 1990; Edmonds *et al.* 1995), these problems have not generally proved sufficiently severe to prevent useful conclusions from being drawn (e.g. Mishina *et al.* 1986).

The obvious way to test the hypothesis that post-translational modification of neuronal nicotinic receptors is defective in oocytes would be to express the receptors in mammalian cell lines. Unfortunately, there are few reports of successful transient or stable transfection of rat neuronal nicotinic receptors into mammalian cell lines (and we have not yet succeeded in attaining sufficient functional expression to allow electrophysiological analysis). None of these studies have attempted single-channel conductance measurements. The only study on $\alpha3\beta4$ receptors showed macroscopic pharmacological properties similar to those of oocyte-expressed $\alpha3\beta4$ (Wong *et al.* 1995).

Another possibility is that, in order to achieve assembly of neuronal nicotinic subunits into a receptor with the correct stoichiometry and topology, native cells use regulatory mechanisms which are absent in oocytes. This does not appear to be such a serious problem for muscle nicotinic subunits, whose correct assembly is driven by the sequences of the N-terminal and, to a lesser extent, the C-terminal domains (Yu & Hall, 1991). If subunit sequence is not sufficient to ensure full subunit–subunit recognition and correct receptor assembly in the case of neuronal nicotinic receptors in oocytes, the consequence would be that the

oocytes would express a 'mosaic' of different receptors. Some evidence that this may indeed be the case comes from the work of Papke, Boulter, Patrick & Heinemann (1989), who have shown that the single-channel conductance of the pairwise combination $\alpha 2\beta 2$ depends on the $\alpha:\beta$ ratio of the cRNA injected, implying that the number of α copies in the pentameric receptor is not necessarily always two.

The situation is even more complicated if we consider 'triplet' combinations. Even if we assume that the $\alpha:\beta$ ratio in the receptor is 2 : 3, and that the order of subunits around the pore is $\alpha\beta\alpha\beta\beta$, injection of $\alpha 3\beta 2\beta 4$ can, in principle, result in four different stoichiometries (the $\alpha 3\beta 2$ and $\alpha 3\beta 4$ pairs plus a triplet $\alpha 3(\beta 2)_2\beta 4$ and a triplet $\alpha 3\beta 2(\beta 4)_2$; each of these triplets can exist in 3 different subunit arrangements, for a total of 8 receptor types). In the case of the injection of $\alpha 3\alpha 5\beta 4$, we could expect one pair ($\alpha 3\beta 4$) and, if $\alpha 5$ substitutes for a β subunit, two possible triplet combinations, $\alpha 3(\alpha 5)_2\beta 4$ and $\alpha 3\alpha 5(\beta 4)_2$ (each of these triplets existing in 3 different subunit orders around the pore). Under these conditions, receptor identification by single-channel recording would be severely hampered by the problem of sampling the receptor mosaic. There are certainly many signs in our results that results can differ from one patch to another, which would be consistent with this view. Taking the case of $\alpha 5$, if the oocyte produces a random mosaic of one pair and two triplet receptors with three different subunit arrangements around the central pore and if only one of these seven receptor types has the right conductance, we can expect that, assuming we sample only one receptor in each patch, an average of fifteen patches will be required before we can be 90% sure of sampling all of the receptor types in the mosaic. It is, therefore, intriguing that with $\alpha 3\alpha 5\beta 4$ we found one patch out of fourteen that showed (among others) a ganglionic-like channel. Clearly, there may be more constraints during assembly that could result in preferential assembly of $\alpha 5$-containing combinations, but these do not appear to be absolute, in the case of $\alpha 5$-containing triplets. Recent work (Ramirez-Latorre *et al.* 1996; Wang *et al.* 1996) indicates that in oocytes injected with either $\alpha 4\alpha 5\beta 2$ or $\alpha 3\alpha 5\beta 4$ combinations, 40 and 45%, respectively, of the receptors do not contain $\alpha 5$ (even if the ratio of cRNAs used for injection is 1 : 10 $\alpha 4\beta 2:\alpha 5$ for the first of these triplet combinations).

Heterogeneity of native neuronal nicotinic receptors

In rat SCG the most prevalent conductance value varied considerably from patch to patch around a mean of 36 pS. The simplest explanation for this finding is that the SCG receptor population is composed of several receptor subtypes which have similar, but not identical conductances. The contribution of lower conductance levels to channel activity varied enormously from one patch to another. Infrequent direct transitions were seen between lower conductance levels and the main 36 pS level, so at least some individual channel types can show more than one conductance level. It is, therefore, not possible to be sure whether heterogeneity results from different channel types spending different proportions of time in the subconductance states, or whether there are also purely low conductance channel types present. In either case, the implication is that the cell is making more than one sort of channel. While we could see no difference in the agonist sensitivity of the different conductances, support for the existence of heterogeneity in sympathetic neurones comes from the observation that clusters of openings from desensitized SCG nicotinic receptors differ in open probability (each cluster is likely to represent the activity of a different receptor molecule; Mathie *et al.* 1991). Furthermore, the biochemical work of Berg and colleagues in chick ciliary ganglia (Corriveau & Berg, 1993; Vernallis *et al.* 1993; Conroy & Berg, 1995) and of Lindstrom and colleagues (Wang *et al.* 1996) in human neuroblastoma SH-SY5Y cells shows that sympathetic neurones can maintain more than one population of nicotinic receptors, which differ in subunit composition.

While our initial question concerning the subunit composition of the ganglion nicotinic receptor cannot be answered, it is clear that none of the receptor subtypes prevalent in SCG neurones is efficiently reproduced by pair or triplet subunit combination in oocytes. It will be important to achieve functional expression of such combinations in mammalian cell lines in order to ascertain the possible contribution or failings of the oocyte expression system. Expression and characterization of receptors of known subunit composition remains a prerequisite to finding out what (if any) is the physiological relevance of receptor heterogeneity in neurones.

Note added in proof

Since this paper was submitted, a paper has appeared by E. Stetzer, U. Ebbinghaus, A. Storch, L. Poteur, A. Schrattenholz, G. Kramer, C. Methfessel & A. Maelicke (*FEBS Letters* **397**, 39–44 (1996)) which describes the successful expression of rat $\alpha 3$ and $\beta 4$ subunits in HEK cells. They find a single-channel conductance of 29 pS in 2 mM calcium. Since the single-channel conductance is decreased by external calcium, it is likely that this value is not very different from the value found in SCG neurones (37 pS in 1 mM Ca^{2+}, this paper; Mathie *et al.* 1991), but it is clearly very different from the 22 pS (in 1 mM Ca^{2+}) found here for $\alpha 3\beta 4$ in oocytes. This new work, therefore, lends weight to the probability that the *Xenopus* oocyte does not reproduce well the characteristics of the native receptor.

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