Stimulation of a nicotinic ACh receptor causes depolarization and activation of L-type Ca^{2+} channels in rat pinealocytes

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1. Membrane voltage (V_m) recordings were obtained from isolated rat pinealocytes using the patch-clamp technique. In parallel to the electrophysiological experiments, intracellular Ca^{2+} measurements were performed using fura-2.

2. The resting V_m averaged −43 mV and replacement of extracellular NaCl by KCl completely depolarized the cells. This indicates that the resting V_m is dominated by a K^+ conductance. Single-channel recordings revealed the presence of a large conductance Ca^{2+}-activated charybdotoxin-sensitive K^+ channel.

3. Application of ACh (100 μM) depolarized the pinealocytes on average by 16 mV. The depolarizing effect of ACh was mimicked by nicotine (50 μM) and was prevented by tubocurarine (100 μM).

4. The ACh-induced depolarization was largely abolished in the absence of extracellular Na^+, but was not significantly affected by extracellular Ca^{2+} removal.

5. Application of ACh (100 μM) caused an increase in $[Ca^{2+}]_i$. This increase was completely dependent on the presence of extracellular Ca^{2+} and was largely reduced after extracellular Na^+ removal. Nifedipine (1 μM) reduced the ACh-induced increase in $[Ca^{2+}]_i$ by about 50%.

6. Our findings indicate that in rat pinealocytes stimulation of a nicotinic ACh receptor (nAChR) induces depolarization mainly by Na^+ influx via the nAChR. The depolarization then activates L-type Ca^{2+} channels, which are responsible for the nifedipine-sensitive portion of the intracellular Ca^{2+} increase. Ca^{2+} influx via the nAChR probably also contributes to the observed rise in $[Ca^{2+}]_i$.

The mammalian pineal organ transduces information about the ambient photoperiod into a neuroendocrine message by rhythmic synthesis and secretion of melatonin (for review see Arendt, 1995). In mammals the sympathetic innervation of the pineal organ is mandatory to maintain the light-synchronized circadian rhythm of melatonin production that is highest during the second half of the night. In rats, noradrenaline acts upon melatonin biosynthesis by stimulating both α_1- and β_1-adrenergic receptors. Stimulation of β_1-receptors increases intracellular cAMP, which mediates the activation of serotonin-*N*-acetyl-transferase, the rate-limiting enzyme of melatonin biosynthesis. Activation of α_1-receptors potentiates the β_1-adrenergic effect via an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (for review see Klein, 1985). Thus, the essential role of noradrenaline for stimulation of melatonin biosynthesis has been firmly established. In contrast, it is still unclear whether and how neuronal pathways other than the noradrenergic sympathetic innervation (for review see Korf, 1996) are involved in the regulation of melatonin biosynthesis and pineal functions in mammals.

Several morphological investigations point toward the existence of a parasympathetic innervation of the mammalian pineal gland, which may originate from the pterygopalatine ganglion and employ acetylcholine as primary neurotransmitter (for review see Møller, 1992). Further evidence for a cholinergic innervation of the pineal organ has been obtained by recent immunocytochemical studies (E. Weihe, M. K.-H. Schäfer & L. E. Eiden, personal communication) using antibodies against the vesicular ACh transporter (Weihe, Tao-Cheng, Schäfer, Erickson & Eiden, 1996). Possible effects of cholinergic agonists on melatonin production and release have been repeatedly investigated (for review see Laitinen, Laitinen & Kokkola, 1995), but the data are equivocal with regard to the receptor types

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involved, their location and the functional consequences of their activation. Transpineal *in vivo* microdialysis has shown that infusion of the cholinergic agonists carbachol and oxotremorine into the rat pineal organ resulted in a marked decrease of melatonin release during the dark period by inhibiting the noradrenaline release from the sympathetic nerve fibres. Such data suggest the presence of muscarinic receptors in a presynaptic location, i.e. on the sympathetic nerve endings (Drijfhout, Grol & Westerink, 1996). The existence of nicotinic ACh receptors (nAChRs) in the pineal organ was inferred from immunocytochemical investigations (Reuss, Schröder, Schröder & Maelicke, 1992) and binding studies with radiolabelled specific ligands (Stankov, Cimino, Marini, Lucini, Fraschini & Clementi, 1993) and it has been suggested that nicotine has an inhibitory effect on pineal melatonin synthesis. Recently ACh has been shown to induce an increase in $[Ca^{2+}]_i$ in isolated rat pinealocytes which is mediated via nAChRs. Nearly all cells, which were identified as pinealocytes by immunocytochemical demonstration of S-antigen, a marker for pinealocytes (arrestin), responded to both noradrenaline and ACh stimulation (Schomerus, Laedtke & Korf, 1995). This suggests that the vast majority of rat pinealocytes express nAChRs.

The goal of the present study was to characterize in detail the membrane potential response of rat pinealocytes to ACh by applying the patch-clamp technique to an *in vitro* system characterized previously (Schomerus *et al.* 1995). In parallel to the electrophysiological experiments, intracellular Ca^{2+} measurements were performed to confirm the responsiveness of the isolated cells and to further elucidate the type of Ca^{2+} response mediated by the nAChR. The effects of ACh were compared with those elicited by noradrenaline. In the course of our study we also characterized the resting K^+ conductance of the cells and identified a Ca^{2+}-activated K^+ channel in single-channel recordings.

METHODS

Animals and treatments

All experiments were conducted in accordance with guidelines on the care of experimental animals as approved by the European Communities Council Directive (86/609/EEC). Adult male Wistar rats were anaesthetized by 4% halothane inhalation and immediately decapitated in the morning between 8.00 and 12.00 h. Pineal glands were rapidly removed and incubated in ice-cold Earle's balanced salt solution (Gibco).

Preparation of dispersed rat pinealocytes

Pineal glands were dissociated by papain digestion and repetitive pipetting as described previously (Schaad, Parfitt, Russell, Schaffner, Korf & Klein, 1993; Schomerus *et al.* 1995). After removal of protease, the dispersed pinealocytes were resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with fetal calf serum (10% v/v), Hepes (10 mM), ascorbic acid (100 μg ml^{-1}), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), glutamine (2 mM) and glucose (7 mg ml^{-1}), seeded onto small pieces of PepTite 2000-coated glass coverslips and incubated at 37 °C in a 5% CO_2 atmosphere. Cells were used 1–3 days after seeding and patch-clamp recordings were obtained from single cells which had no contact with neighbouring cells.

Patch-clamp technique

The inside-out and outside-out configuration of the patch-clamp technique was used (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) to record single-channel currents. To continuously monitor the membrane voltage (V_m) in whole-cell recordings the ruptured patch or the perforated patch configuration (Horn & Marty, 1988) was used. For perforated patch recordings the pipettes were back-filled with a pipette solution containing 120 μg ml^{-1} amphotericin B, which was added from a 60 mg ml^{-1} amphotericin B stock solution in DMSO. Perforated patch recordings were usually more stable than conventional ruptured patch recordings. However, both techniques revealed very similar electrophysiological responses of the cells. Thus, the results from these experiments were pooled and are reported together. Experiments were performed at room temperature (18–22 °C) and experimental procedures were as described previously (Letz, Ackermann, Canessa, Rossier & Korbmacher, 1995). Patch pipettes were pulled from Clark glass capillaries (Clark Electromedical Instruments, Pangbourne, UK). In NaCl solution the resistance (R_{pip}) of pipettes filled with potassium gluconate–KCl solution (see below) averaged 5·9 ± 0·3 MΩ ($n = 68$). Currents were amplified with an EPC-9 patch-clamp amplifier (HEKA elektronik, Lambrecht, Germany). Membrane capacitance (C_m) and series resistance (R_s) were estimated by nulling capacitive transients using the automated EPC-9 compensation circuit. In the conventional ruptured patch configuration C_m and R_s averaged 12·4 ± 1·5 MΩ and 8·7 ± 0·9 pF ($n = 36$), respectively. The reference electrode was an Ag–AgCl pellet bathed in the same solution as that in the pipette and connected to the bath via an agar–pipette solution bridge in the outflow path of the chamber. Liquid junction potentials occurring at the bridge–bath junction were measured using a 3 M KCl-flowing boundary electrode and ranged from −3 to +9 mV. The pipette potential (V_{pip}) settings and V_m values were corrected accordingly while the original traces shown in the figures are not corrected for the liquid junction potential. Upward current deflections correspond to cell membrane outward currents, i.e. movement of positive charge from the cytoplasmic side to the extracellular side. Single-channel current amplitudes for *I–V* plots were estimated from amplitude histograms or by measuring and averaging several individual current transitions. Channel activity was estimated from binned current amplitude histograms as the product NP_o, where N is the number of channels and P_o is single-channel open probability. The computer software for data analysis was written by A. Rabe in our laboratory. Current data were recorded at 10 kHz bandwidth. For single-channel analysis, currents were filtered at 1 kHz using an 8-pole Bessel filter and sampled at a rate of 5 kHz. Data are given as mean values ± S.E.M. and significances were evaluated by the appropriate version of Student's *t* test.

Solutions and chemicals

For the patch-clamp experiments the standard bath solution was a NaCl solution (mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes; adjusted to pH 7·5 with NaOH. Other bath solutions were a KCl solution (mM: 140 KCl, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes; adjusted to pH 7·5 with KOH) and an NMDG-Cl solution (mM: 145 *N*-methyl-D-glucamine-Cl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes; adjusted to pH 7·5 with Tris). Bath solutions with different K^+ concentrations were obtained by replacing 5, 10, 20, 40 or 80 mM NMDG-Cl with equimolar KCl. Ca^{2+}-free bath solutions

were obtained by omitting $CaCl_2$ and by adding 1 mM EGTA. All bath solutions contained 5 mM glucose. The pipette solution was a potassium gluconate–KCl solution (mM: 115 potassium gluconate, 25 KCl, 5 NaCl, 1 $MgCl_2$, 0·58 $CaCl_2$ and 1 EGTA to give a free Ca^{2+} concentration of 10^{-7} M, 10 Hepes; adjusted to pH 7·5 with KOH). Charybdotoxin, ACh, nifedipine and noradrenaline hydrochloride were obtained from Sigma. ACh and noradrenaline solutions were prepared freshly on the day of the experiment. The noradrenaline solution was supplemented with 100 μM ascorbic acid and was kept in the dark.

Fluorescence microscopy

Using the calcium-sensitive fluorescent dye fura-2, $[Ca^{2+}]_i$ was monitored in isolated pinealocytes from the same cell preparations as used for the patch-clamp experiments. Dye loading of pineal cells with fura-2 and fluorescence microscopy using an Attofluor ratio imaging system (Zeiss) were performed as described previously (Schomerus *et al.* 1995). Excitation light was provided by a mercury lamp (HBO 100 W/2; Osram GmbH, Berlin-München, Germany); excitation wavelengths of 334 and 380 nm were selected by interference filters mounted on a computer-controlled filter changer. Fluorescent light was collected by an Achrostigmat × 40 oil-immersion objective (Zeiss), passed through a dichroic mirror (395 nm) and an emission filter (500–530 nm) and finally transmitted to a CCD camera with a photomultiplier. The velocity of data aquisition for one 334 and 380 nm image pair was 2 s at a resolution of 512 × 512 pixels per image. Each experimental protocol was performed on at least three different batches of cells and in each experiment fluorescence measurements were obtained from nineteen to forty-three regions of interest corresponding to individual cells. In some experiments ratio data were converted to approximate calcium concentrations by performing *in vivo* calibrations as described previously (Schomerus *et al.* 1995) using the equation of Grynkiewicz, Poenie & Tsien (1985).

RESULTS

Continuous membrane voltage recordings in isolated rat pinealocytes

In isolated rat pinealocytes gigaohm seals were obtained in about one out of three attempts. Following gigaohm seal formation the whole-cell configuration was achieved in about 50% of the attempts; the seals could be maintained for up to 1 h. This made it possible to perform solution exchanges and to apply drugs while continuously monitoring V_m in zero current-clamp mode. The initial resting V_m in NaCl bath solution averaged $-42{\cdot}9 \pm 1{\cdot}7$ mV ($n = 72$) and complete replacement of bath NaCl by KCl reversibly depolarized the cells to $1{\cdot}9 \pm 0{\cdot}6$ mV ($n = 70$). This indicates that the resting V_m of the isolated pinealocytes is dominated by a K^+ conductance. The responsiveness to KCl exposure was routinely tested to verify the validity of the membrane voltage recording during an experiment.

K^+ conductance of rat pinealocytes

In experiments like that shown in Fig. 1*A* we further investigated the K^+ conductance in isolated rat pinealocytes. To eliminate any contribution of a Na^+ conductance these experiments were carried out in the absence of extracellular Na^+, which was replaced by the non-permeating cation NMDG (*N*-methyl-D-glucamine). Step changes of the bath K^+ concentration from 5 to 10, 20, 40, 80 or 145 mM immediately and reversibly depolarized the cell membrane in a concentration-dependent way. Results from seven similar experiments are summarized in Fig. 1*B*. Membrane voltage is plotted *versus* the logarithm of the extracellular

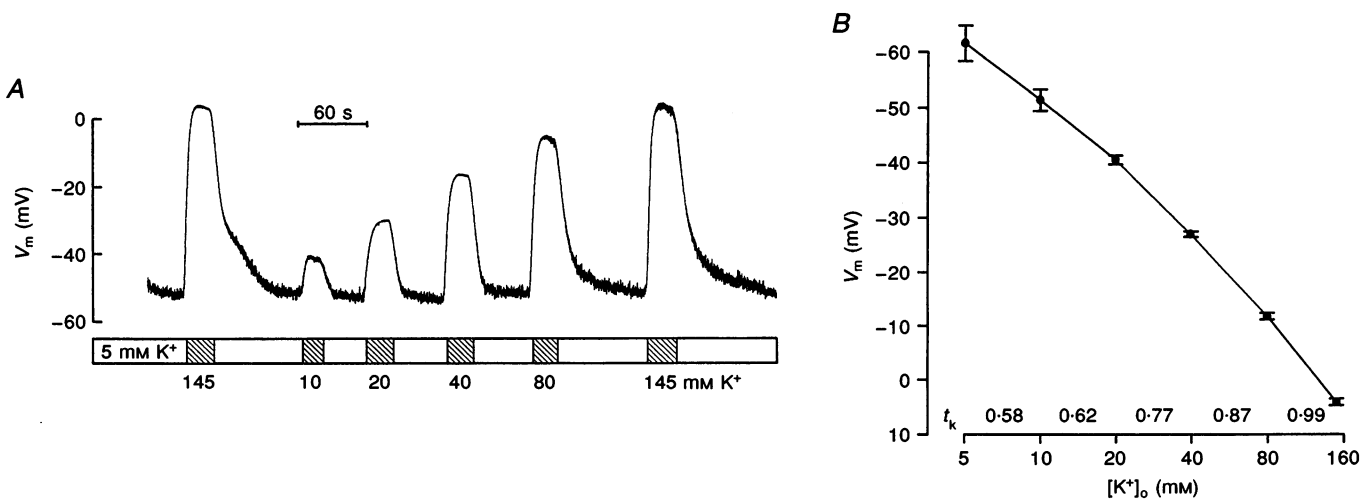

Figure 1. Extracellular K^+ depolarizes pinealocytes in a concentration-dependent way

The experiments were carried out in the absence of extracellular Na^+, which was replaced by the non-permeating cation NMDG (*N*-methyl-D-glucamine). *A*, during a continuous membrane voltage (V_m) recording from an individual pinealocyte bath K^+ concentration was changed from 5 to 10, 20, 40, 80 or 145 mM, as indicated below the trace. *B*, results from 7 experiments similar to that shown in *A* are summarized, and the average V_m is plotted *versus* the extracellular K^+ concentration ($[K^+]_o$) using a logarithmic scale. Transference numbers (t_k) were calculated from the linear slopes between adjacent data points.

K^+ concentration ($[K^+]_o$). From a Nernstian behaviour one would expect a linear relation. However, the curve is slightly steeper at higher K^+ concentrations, with a slope of 57·6 mV per decade $[K^+]_o$ for concentrations between 80 and 145 mM compared with a slope of 33·7 mV per decade $[K^+]_o$ for concentrations between 5 and 10 mM. The corresponding relative K^+ conductances, expressed as potassium transference numbers (t_k, observed slope *versus* Nernst slope), were 0·99 and 0·58, respectively.

Identification and characterization of K^+ channels in outside-out and inside-out patches

After achieving the ruptured patch whole-cell configuration outside-out patches were obtained by drawing the pipette tip away from the cell surface. The single-channel currents shown in Fig. 2*A* were recorded at different holding potentials from an outside-out patch bathed in KCl solution. A single-channel *I–V* plot derived from these current traces is shown in Fig. 2*B* (○). Under asymmetrical conditions for Cl^-, with potassium gluconate–KCl solution in the pipette and KCl solution in the bath, the measured reversal potential of close to 0 mV indicates that the single-channel currents are carried by K^+. A linear fit of the data revealed a single-channel conductance of 202 pS. In ten similar recordings the single-channel conductance averaged 216 ± 7 pS. The other *I–V* plot in Fig. 2*B* (●) was derived from current transitions observed in the same outside-out patch after changing from KCl to NaCl bath solution. In the presence of NaCl bath solution no more inward current transitions could be observed and the reversal potential estimated from a Goldman–Hodgkin–Katz fit of the outward current data was shifted far to the left. Taken together the two *I–V* curves indicate that the observed single channels are K^+ selective. As can be seen in Fig. 2*A*, channel activity increases with depolarization. This is illustrated in Fig. 2*C*, in which NP_o is plotted *versus* the holding potential. This voltage dependence is compatible with the finding that the relative K^+ conductance increases at higher extracellular K^+ concentrations. At high extracellular K^+ concentrations the cells are depolarized and therefore the channels are more active.

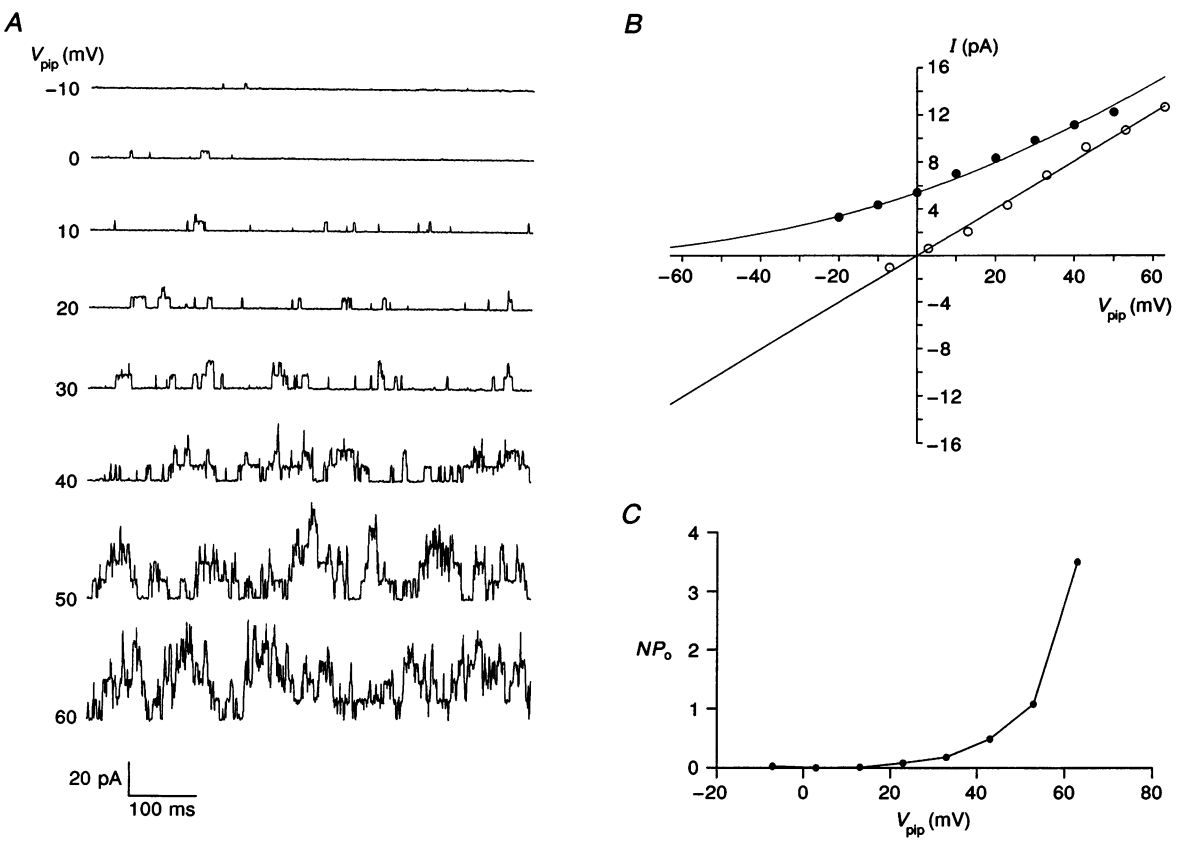

Figure 2. Large conductance K^+ channel in an outside-out patch

A, single-channel current traces from an outside-out patch held at different pipette potentials (V_{pip}). The pipette solution was potassium gluconate–KCl and the bath solution was KCl. *B*, single-channel *I–V* plots obtained from the same patch as shown in *A*, first in KCl (○) and subsequently in NaCl (●) bath solution. *C*, channel activity, expressed as the product NP_o (where N is the number of channels and P_o is single-channel open probability) is plotted *versus* V_{pip}. Data were obtained in KCl bath solution from the same patch as shown in *A*.

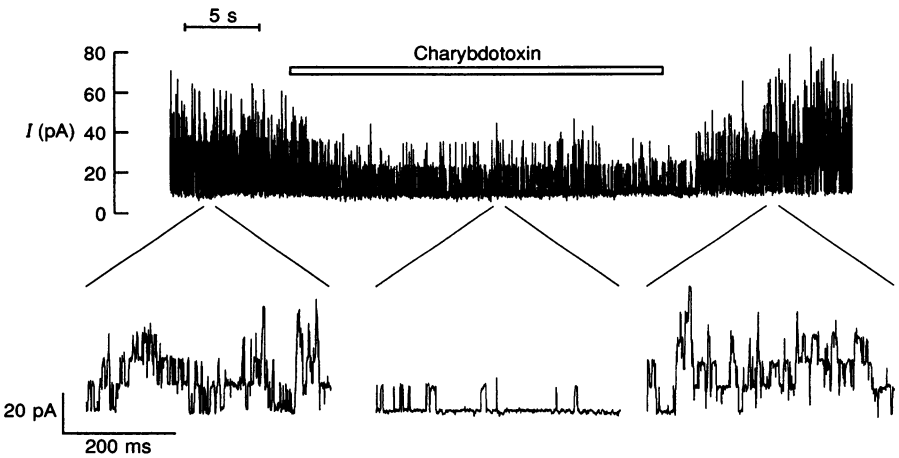

Figure 3. Charybdotoxin inhibits the large conductance K^+ channel

A continuous current trace recorded from an outside-out patch using a V_{pip} of 60 mV is shown. Charybdotoxin was applied at a concentration of 100 nM as indicated. Insets below show the indicated portions of the current trace on an expanded time axis. Pipette solution, potassium gluconate–KCl.

Figure 3 illustrates the effect of the K^+ channel blocker charybdotoxin on K^+ channel activity recorded from an outside-out patch. Application of 100 nM charybdotoxin reversibly reduced NP_o by 83·4 ± 5·4% ($n = 4$). Charybdotoxin is known to block Ca^{2+}-activated maxi K^+ channels (for review see Garcia, Knaus, Munujos, Slaughter & Kaczorowski, 1995). Therefore the cytoplasmic Ca^{2+} dependence of channel activity was tested in excised inside-out patches. As demonstrated by the inside-out patch recording shown in Fig. 4, removal of cytoplasmic Ca^{2+} practically abolished K^+ channel activity, which resumed after re-addition of Ca^{2+}.

Membrane voltage response to ACh and noradrenaline

If the response of rat pinealocytes to a neurotransmitter involves the activation or inhibition of ion channels, the application of this neurotransmitter should result in a corresponding change of the membrane potential. We therefore tested the effect of ACh and noradrenaline on the membrane potential of isolated rat pinealocytes. As shown in Fig. 5, application of 100 μM ACh depolarized the pinealocyte. In contrast, in the same experiment application of 1 μM noradrenaline had no effect. Step changes from NaCl to KCl bath solution reversibly depolarized the cell and confirmed the validity and stability of the membrane

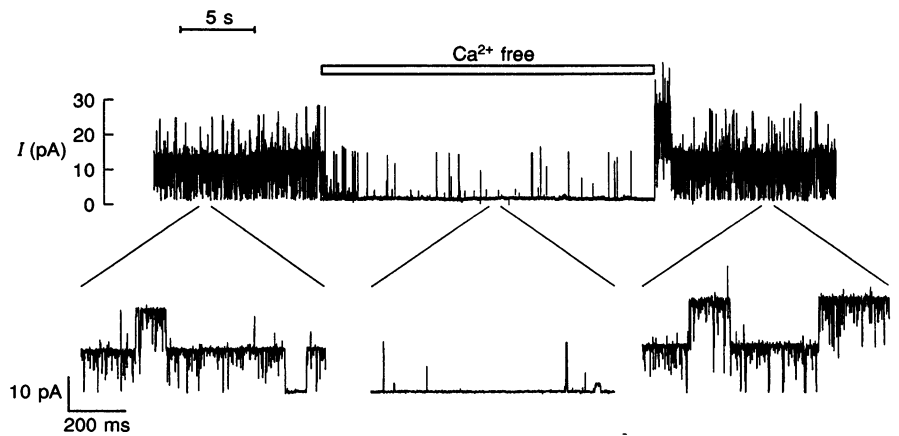

Figure 4. Ca^{2+} sensitivity of the large conductance K^+ channel

A continuous current trace recorded from an inside-out patch using a V_{pip} of −40 mV is shown. Ca^{2+} was removed from the cytoplasmic side of the patch as indicated. Insets below show the indicated portions of the current trace on an expanded time axis. Pipette solution, potassium gluconate–KCl.

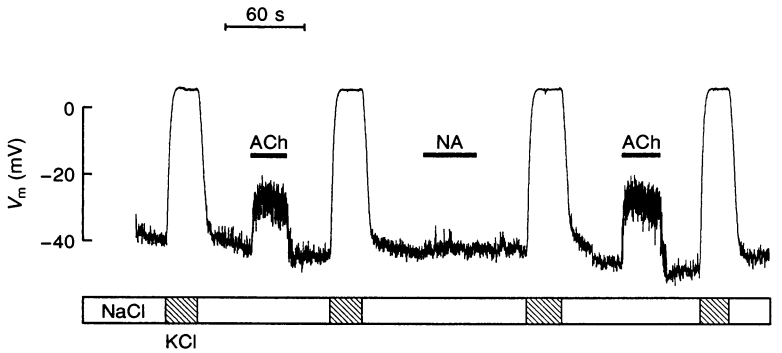

Figure 5. ACh depolarizes pinealocytes while noradrenaline has no effect

During a continuous V_m recording from an individual pinealocyte ACh (100 μM) and noradrenaline (NA; 1 μM) were applied as indicated. The bath solution was repeatedly changed from NaCl to KCl solution as indicated below the trace; pipette solution, potassium gluconate–KCl.

voltage recording throughout the experiment. The ACh-induced depolarization persisted until ACh was washed out. The depolarizing response could be elicited several times in the same cell without apparent desensitization. The magnitude of the depolarization ranged from 2 to 46 mV in different cells and averaged 15·6 ± 1·4 mV ($n = 40$). An effect of ACh was observed in forty out of fifty-one pinealocytes investigated. In contrast, in nine experiments using the ruptured patch configuration ($n = 9$) and in ten experiments using the perforated patch configuration noradrenaline, at concentrations of 1 or 10 μM, had no significant effect. In these experiments membrane voltage averaged −45·2 ± 4·0 and −45·5 ± 4·1 mV in the presence and absence of noradrenaline, respectively ($n = 19$).

The ACh response is mediated by a nicotinic receptor

As shown in Fig. 6, application of 50 μM nicotine had a similar depolarizing effect to the application of 100 μM ACh. In two such experiments nicotine was applied three times and induced an average depolarization of 33·7 ± 9·6 mV. In the same experiments ACh depolarized the cells by 28·5 mV. In the presence of 100 μM tubocurarine, a competitive inhibitor of the nAChR, the depolarizing response to 100 μM ACh was largely abolished ($n = 7$; Fig. 6). These findings indicate that in rat pinealocytes the membrane voltage response to ACh is mediated by a nAChR. Nicotinic ACh receptors are ligand-gated non-selective cation channels and, under physiological conditions, mediate Na^+ influx upon activation by ACh. To test whether, in pinealocytes, Na^+ influx is responsible for the ACh-induced depolarization, experiments were performed in which ACh was applied in the absence of extracellular Na^+. Complete replacement of extracellular Na^+ by the non-permeating cation NMDG usually caused a significant hyperpolarization, which averaged −25·3 ± 3·5 mV ($n = 9$) and ranged from −12 to −46 mV. This suggests the presence of a Na^+ conductance or of an electrogenic Na^+–Ca^{2+} exchanger in the pinealocytes (see below). In the present study this hyperpolarizing effect of Na^+ removal was not investigated further. As illustrated in Fig. 7, application of ACh no longer depolarized the pinealocytes in the absence of extracellular Na^+. During Na^+ removal the membrane voltage averaged −60·0 ± 5·1 mV

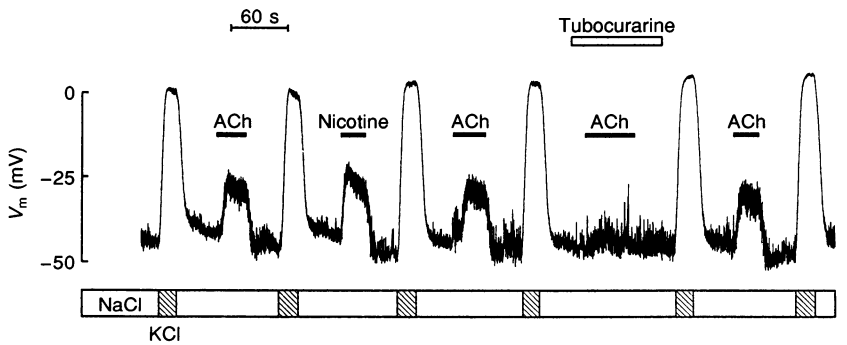

Figure 6. Nicotine mimics the depolarizing effect of ACh while tubocurarine prevents the response to ACh

During a continuous V_m recording the effect of nicotine (50 μM) was tested and compared to the effect of ACh (100 μM). Furthermore, ACh was applied in the presence of tubocurarine (100 μM). The bath solution was repeatedly changed from NaCl to KCl solution as indicated below the trace.

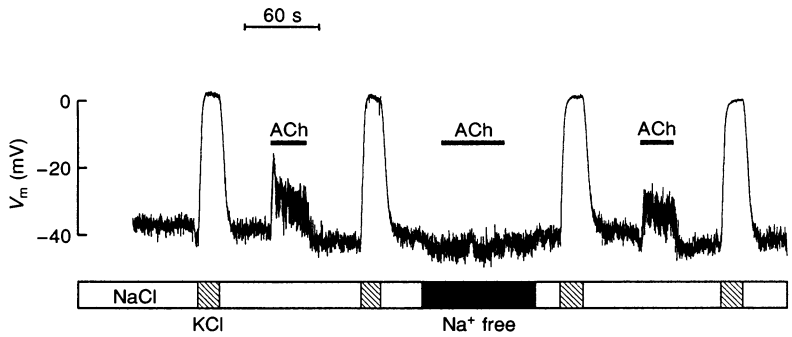

Figure 7. ACh-induced depolarization depends on extracellular Na^+

The effect of ACh (100 μM) was tested in NaCl bath solution and in Na^+-free bath solution in which Na^+ was replaced by the non-permeating cation NMDG. The V_m trace is not corrected for the liquid junction potential of 9 mV that occurs upon changing from NaCl to NMDG-Cl bath solution. Considering this liquid junction potential the actual hyperpolarization induced by changing to Na^+-free bath solution was −12 mV in this particular experiment. Bath solution was repeatedly changed from NaCl to KCl solution as indicated below the trace.

before and −58·6 ± 4·8 mV after the application of 100 μM ACh ($n = 9$; not significantly different). In contrast, in the same experiments, application of ACh in the presence of extracellular Na^+ had the usual effect and depolarized the cells by 14·3 ± 2·4 mV ($n = 9$). This indicates that ACh activates a depolarizing Na^+ inward current, as expected during the activation of nAChRs.

Role of extracellular calcium for the membrane voltage response to ACh

Some subtypes of nAChRs are known to have a considerable Ca^{2+} permeability (Mulle, Choquet, Korn & Changeux, 1992; Vernino, Amador, Luetje, Patrick & Dani, 1992) and intracellular Ca^{2+} measurements have previously shown that in rat pinealocytes the increase in $[Ca^{2+}]_i$ upon application of ACh depends on the presence of extracellular Ca^{2+} (Schomerus *et al.* 1995). Therefore, it was tested whether the depolarizing response to ACh was dependent on the presence of extracellular Ca^{2+} and whether it was affected by nifedipine, a blocker of L-type voltage-gated Ca^{2+} channels. As demonstrated by the recording shown in Fig. 8, ACh elicited a normal depolarization in the presence of 1 μM nifedipine and also in the absence of extracellular Ca^{2+}. In similar experiments the ACh-induced depolarization averaged 15·3 ± 3·8 and 18·1 ± 3·1 mV ($n = 8$; not significantly different) in the presence and absence, respectively, of 1 μM nifedipine. It averaged 14·8 ± 1·2 mV in the presence and 11·6 ± 2·8 mV in the absence of extracellular Ca^{2+} ($n = 5$; not significantly different). Thus, the ACh-induced depolarization was not significantly affected by nifedipine or extracellular Ca^{2+} removal.

Intracellular Ca^{2+} measurements

As reported previously, isolated rat pinealocytes respond to both ACh and noradrenaline with an increase in $[Ca^{2+}]_i$ (Schomerus *et al.* 1995). Typical Ca^{2+} responses to ACh and

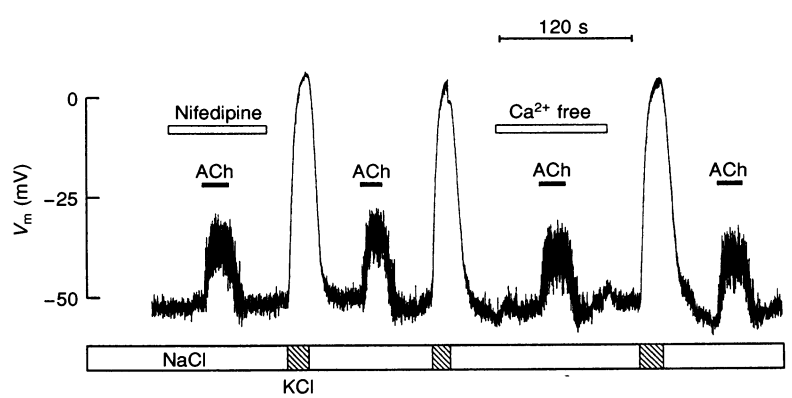

Figure 8. ACh-induced depolarization is not significantly reduced in the presence of nifedipine or in the absence of extracellular Ca^{2+}

A continuous V_m recording is shown during which ACh (100 μM) was applied several times and its effect was tested in the presence of nifedipine (1 μM) and in the absence of extracellular Ca^{2+}. Bath solution was repeatedly changed from NaCl to KCl solution as indicated below the trace.

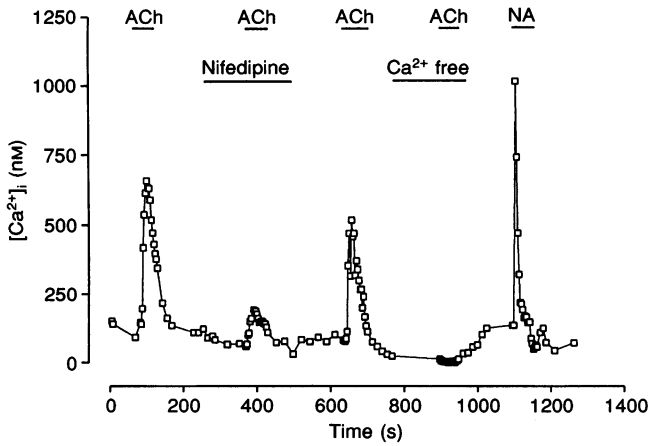

Figure 9. ACh-induced increase in $[Ca^{2+}]_i$ is partially blocked by nifedipine and is abolished in the absence of extracellular Ca^{2+}

$[Ca^{2+}]_i$ was monitored in fura-2-loaded pinealocytes. Data points represent mean values from 19 individual cells. ACh was repeatedly applied at a concentration of 100 μM. Nifedipine was used at a concentration of 1 μM. Extracellular Ca^{2+} was removed as indicated (Ca^{2+} free). At the end of the experiment NA was applied at a concentration of 1 μM.

noradrenaline are shown in Fig. 9 and were consistently observed in several different preparations of isolated rat pinealocytes used in the present study. This demonstrates that the absence of an electrophysiological response to noradrenaline does not mean that the studied cells were intrinsically unresponsive to noradrenaline. Whereas nifedipine or extracellular Ca^{2+} removal had no effect on the depolarization induced by ACh, 1 μM nifedipine reduced the intracellular Ca^{2+} response to ACh on average by $49 \pm 4\%$ ($n = 4$; $P < 0{\cdot}01$) and extracellular Ca^{2+} removal abolished it ($n = 8$; Fig. 9). This indicates that the ACh-induced intracellular Ca^{2+} increase is not primarily mediated by Ca^{2+} release from intracellular stores but depends on extracellular Ca^{2+} and involves the activation of a nifedipine-sensitive Ca^{2+} entry mechanism. Nifedipine is a known blocker of voltage-activated L-type Ca^{2+} channels. Experiments like those shown in Fig. 10 demonstrated that increasing the extracellular K^+ concentration to 10, 20, 40 and 80 mM increased the $[Ca^{2+}]_i$ in a concentration-dependent manner ($n = 5$). Since increasing the extracellular K^+ concentration depolarizes the pinealocytes (see above), these findings suggest that activation of voltage-dependent Ca^{2+} channels may be responsible for the intracellular Ca^{2+} increase. This is supported by the finding that the intracellular calcium increase evoked by 40 mM KCl was reduced by $26 \pm 2\%$ ($n = 3$) and $65 \pm 2\%$ ($n = 4$) in the presence of 1 and 10 μM nifedipine, respectively. The magnitude of the intracellular Ca^{2+} increase observed upon application of 100 μM ACh was intermediate to that observed with 20 and 40 mM K^+ (Fig. 10).

If the ACh-induced Ca^{2+} increase is mediated by depolarization, ACh should not elicit a Ca^{2+} response in the absence of extracellular Na^+ since this prevents the ACh-induced depolarization (see above). As illustrated in Fig. 11, the Ca^{2+} response to ACh is indeed largely dependent on the presence of extracellular Na^+. Simultaneous removal of extracellular Ca^{2+} and Na^+ slightly decreased the $[Ca^{2+}]_i$ and, as expected, application of ACh had virtually no effect (Fig. 11). Re-addition of extracellular Ca^{2+} in the absence of Na^+ caused an increase in $[Ca^{2+}]_i$ above baseline. This is compatible with the presence of a Na^+–Ca^{2+} exchanger in the membrane of the pinealocytes (see above). The Na^+–Ca^{2+} exchanger is important in maintaining intracellular Ca^{2+} homeostasis and is known to be present in many cells, including neuronal cells (for reviews see Blaustein *et al.* 1991). In the presence of extracellular Ca^{2+} and absence of extracellular Na^+ the response to ACh was greatly reduced (Fig. 11).

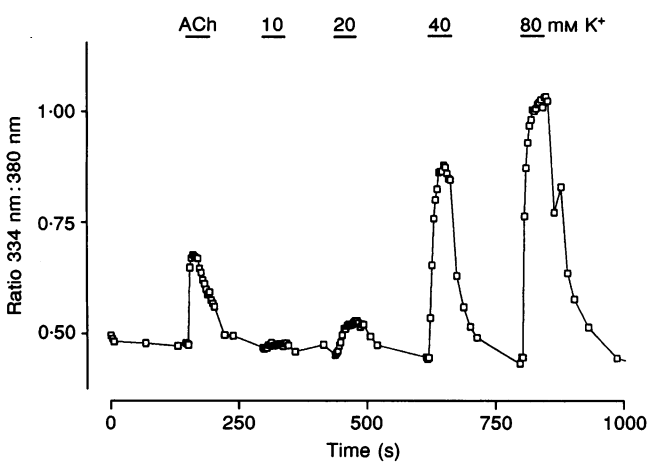

Figure 10. $[Ca^{2+}]_i$ is increased by K^+-induced depolarization

$[Ca^{2+}]_i$ was monitored in fura-2-loaded pinealocytes and is expressed as a fluorescence ratio (334 nm : 380 nm). Data points represent mean values from 31 individual cells. Extracellular K^+ concentration was increased from 5 to 10, 20, 40 and 80 mM as indicated. This was achieved by equimolar replacement of NaCl by KCl in the bath solution. At the beginning of the experiment ACh (100 μM) was applied as indicated.

DISCUSSION

The main finding of the present study is that rat pinealocytes show an electrical response to ACh and that this response is mediated by a nicotinic acetylcholine receptor (nAChR). Moreover, the study demonstrates that the previously described *in vitro* system of isolated rat pinealocytes (Schomerus *et al.* 1995) is suitable for investigating the electrophysiological properties of this tissue.

K^+ conductance of rat pinealocytes

The measured resting membrane potential of −43 mV confirms the viability of the isolated pinealocytes and compares favourably with the −40 mV obtained with conventional intracellular microelectrodes in microdissected rat pineal glands (Sakai, Schneider, Felt & Marks, 1976). Accordingly, under resting conditions the dominating membrane conductance of pinealocytes is a K^+ conductance, and several types of K^+ currents (Aguayo & Weight, 1988; Castellano, Lópes-Barneo & Armstrong, 1989; Aguayo, 1989) as well as a Ca^{2+}-activated maxi K^+ channel (BK_{Ca}) (Halperin & Yeandle, 1987; Ceña, Halperin, Yeandle & Klein, 1991) have previously been identified in dissociated cells from rat pineal gland using the patch-clamp technique. The channel described in this latter study is similar to the large conductance Ca^{2+}-activated charybdotoxin-sensitive K^+ channel identified in the present study, which also displays voltage dependence with an increased channel activity at depolarizing holding potentials. Activation of K^+ channels by depolarization and by an increase in intracellular Ca^{2+} may be an important regulatory mechanism for the maintenance of the resting membrane voltage. Similar so-called BK_{Ca} channels have been described in many different cell types, including neurons in which they are believed to contribute to action potential repolarization (for review see Sah, 1996). In pinealocytes it has been suggested that an increase in open probability of these BK_{Ca} channels (Ceña, Halperin, Yeandle & Klein, 1991) is responsible for the hyperpolarization observed upon application of noradrenaline in the intact pineal gland (Sakai & Marks, 1972) as well as in cultured pinealocytes (Freschi & Parfitt, 1986). In contrast to these studies, we did not detect a hyperpolarizing response to noradrenaline in our cell system while an increase in $[Ca^{2+}]_i$ in response to noradrenaline application was regularly observed (Schomerus *et al.* 1995), indicating that functional adrenergic receptors are present in these cells. Since cell dialysis with a pipette solution may adversely affect the hormone responsiveness of cells in the conventional ruptured patch whole-cell configuration, we also tested noradrenaline using the perforated patch technique, which should leave the intracellular signalling pathways largely intact (Horn & Marty, 1988). However, even under these conditions we did not detect a hyperpolarization upon application of noradrenaline. Interestingly, Freschi & Parfitt (1986) found that in their cultures only 30% of the rat pinealocytes tested showed a hyperpolarizing response to noradrenaline, and in the pineal gland of guinea-pigs application of noradrenaline even caused a depolarization of pinealocytes (Parkington, McCance & Coleman, 1987). At present we do not have an explanation for the complete lack of an electrophysiological response to noradrenaline in our cell system.

Response to ACh is mediated by a nicotinic ACh receptor

In 78% of the pinealocytes tested, application of ACh (100 μM) caused a detectable depolarization that ranged from 2 to 46 mV and averaged about 16 mV. This depolarization persisted in the continuous presence of ACh and was reversible upon washout. The findings that nicotine mimicked the effect of ACh and that the ACh response was inhibited by tubocurarine clearly indicate that the effect of ACh is mediated by a nAChR. Thus, the electrophysiological data from our present study confirm the conclusion previously drawn from corresponding intracellular Ca^{2+} measurements obtained in the same *in vitro* system (Schomerus *et al.* 1995). Numerous nAChR subunits are expressed in the vertebrate nervous system, giving rise to the potential for enormous receptor diversity (for reviews see Sargent, 1993; McGehee & Role, 1995). Neuronal nAChRs are ligand-gated non-selective cation channels with single-channel conductances ranging from 5 to 45 pS. Under

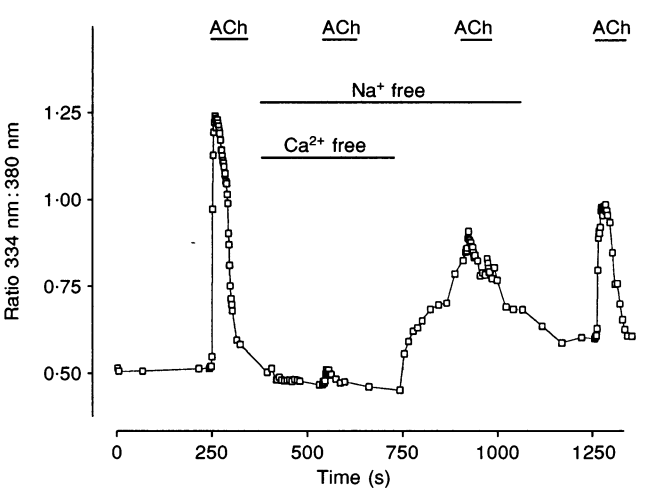

Figure 11. Effect of Na^+ removal on the intracellular Ca^{2+} response to ACh

$[Ca^{2+}]_i$ was monitored in fura-2-loaded pinealocytes and is expressed as a fluorescence ratio (334 nm : 380 nm). Data points represent mean values from 43 individual cells. Extracellular Ca^{2+} and extracellular Na^+ were removed for the periods indicated. ACh was repeatedly applied at a concentration of 100 μM.

physiological conditions the main cation entering the cell via activated nAChRs is Na^+. This is compatible with our finding that the ACh-induced depolarization was practically abolished when extracellular Na^+ was replaced by the impermeant cation NMDG.

Mechanism of Ca^{2+} entry elicited by ACh

As previously reported (Schomerus *et al.* 1995) and confirmed in the present study, in rat pinealocytes the intracellular Ca^{2+} response to ACh is completely abolished in the absence of extracellular Ca^{2+}, indicating that Ca^{2+} entry is essential for the rise in $[Ca^{2+}]_i$. In contrast, the depolarizing response to ACh was practically unaffected by extracellular Ca^{2+} removal, as expected if Na^+ was the main charge-carrying ion mediating the depolarizing response. Most neuronal nAChRs are not exclusively permeable to monovalent cations but are also permeable to Ca^{2+}, and can mediate physiologically significant Ca^{2+} entry (Vernino *et al.* 1992; Mulle *et al.* 1992) even though less than 3% of the total inward current through the nAChR may be carried by Ca^{2+}, as shown in adrenal chromaffin cells (Zhou & Neher, 1993). It has been estimated that calcium-specific currents of only 2 pA can raise levels of intracellular Ca^{2+} at a rate of 100 nM s^{-1} (Neher, 1992). Thus, a very small contribution of Ca^{2+} to the depolarizing inward current via the nAChR may be sufficient to cause a significant increase in $[Ca^{2+}]_i$ in the pinealocytes. However, such a small calcium component is likely to remain undetected in our membrane voltage recordings. Indeed, the depolarization induced by ACh was not significantly altered in the absence of extracellular Ca^{2+}. However, while extracellular Na^+ removal almost completely abolished the depolarizing response to ACh, we still observed a small rise in $[Ca^{2+}]_i$ upon application of ACh in the absence of extracellular Na^+. This is likely to reflect Ca^{2+} entry via the nAChR. On the other hand, the markedly reduced magnitude of the intracellular Ca^{2+} increase in the absence of extracellular Na^+ suggests that in addition to a possible Ca^{2+} entry via the nAChR another Ca^{2+} entry mechanism must exist and is activated following ACh application. Since Na^+ removal prevents the ACh-induced membrane voltage depolarization and reduces the intracellular Ca^{2+} increase, it appears plausible that a depolarization-dependent process, namely a voltage-dependent Ca^{2+} channel, contributes to the rise in $[Ca^{2+}]_i$ after application of ACh. Indeed, we could demonstrate that nifedipine, a known inhibitor of L-type Ca^{2+} channels, reduced the ACh-induced rise in $[Ca^{2+}]_i$ by about 50%. Moreover, increasing the extracellular K^+ concentration, a manoeuvre shown to depolarize the pinealocytes, caused a concentration-dependent increase in $[Ca^{2+}]_i$, which again was partially sensitive to nifedipine. These experiments also suggest the presence of L-type Ca^{2+} channels. The depolarization elicited by ACh was not significantly reduced by nifedipine, which confirms the conclusion that it is mainly mediated by Na^+ influx via the nAChR. The contribution of L-type Ca^{2+} channels to the depolarization is likely to be too small to be detected in our membrane voltage recordings, yet the Ca^{2+} influx mediated by these channels may be physiologically relevant. Indeed, a recent study in isolated smooth muscle cells demonstrating depolarization-evoked increases in $[Ca^{2+}]_i$ via L-type Ca^{2+} channels suggests that about six continuously open 3·5 pS channels would be sufficient to account for the observed intracellular Ca^{2+} rise (Kamishima & McCarron, 1996). Interestingly, in this study the largest increase in $[Ca^{2+}]_i$ occurred at a depolarization to −30 mV, which is well within the membrane voltage range reached in rat pinealocytes upon application of ACh. Recent studies from other laboratories have demonstrated the presence of L-type Ca^{2+} channels in rat pinealocytes (Chin, Smith, Kim & Kim, 1992; Chik, Liu, Girard, Karpinski & Ho, 1992; Chik, Liu, Li, Karpinski & Ho, 1995), which further supports our interpretation that ACh-induced Ca^{2+} influx is in part mediated by activation of L-type Ca^{2+} channels. Moreover, nAChR activation in chromaffin cells (O'Sullivan, Cheek, Moreton, Berridge & Burgoyne, 1989), in ciliary neurons (Rathouz & Berg, 1994) and in rat hippocampal neurons (Barrantes, Murphy, Westwick & Wonnacott, 1995) has also been shown to cause Ca^{2+} influx that results from depolarization and opening of voltage-gated Ca^{2+} channels.

In conclusion, our findings indicate that in rat pinealocytes a nAChR mediates the ACh-induced depolarization and that the depolarization is mainly caused by Na^+ influx via the nAChR. The depolarization then activates L-type Ca^{2+} channels, which are responsible for the nifedipine-sensitive portion of the intracellular Ca^{2+} increase observed upon stimulation by ACh. Ca^{2+} influx via the nAChR itself probably also contributes to the rise in $[Ca^{2+}]_i$. Moreover, Ca^{2+} entering the cell via these two pathways may subsequently trigger Ca^{2+} release from intracellular stores, which may enhance the size of the intracellular Ca^{2+} signal following the application of ACh. The fact that the nAChR is expressed in the vast majority of pinealocytes indicates its important role in the regulation of pineal function. Some studies suggest that stimulation of nAChRs may inhibit melatonin secretion (Stankov *et al.* 1993; Drijfhout *et al.* 1996) but the precise signalling pathways remain elusive (Laitinen *et al.* 1995). One possibility may be that the stimulation of the nAChR and the subsequent activation of the L-type Ca^{2+} channel stimulates release of glutamate, shown to accumulate in microvesicles of the pinealocytes (Redecker & Veh, 1994), and to inhibit melatonin secretion (Kus, Handa & McNulty, 1994). Further experimental clarification of this issue and elucidation of the molecular nature and subunit composition of the pineal nAChR are now needed to understand the interactions between the noradrenergic and cholinergic innervation of the mammalian pineal gland.

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