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Modulation of electrically evoked acetylcholine release in cultured rat septal neurones

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Abstract

The electrically evoked release of acetylcholine and its modulation via auto- and heteroreceptors were studied in primary cell cultures prepared from embryonic rat septum (ED 17). Cultures were grown for 1, 2 or 3 weeks on circular, poly D-lysine-coated glass coverslips. They developed a dense network of non-neuronal and neuronal cells, only some of which were immunopositive for choline acetyltransferase. To measure acetylcholine release, the cells on the coverslips were pre-incubated with [^{3}H]choline (0.1 μmol/L), superfused with modified Krebs–Henseleit buffer at 25°C and electrically stimulated twice for 2 min (S_1, S_2; 3 Hz, 0.5 ms, 90–100 mA). The electrically evoked overflow of [^{3}H] from the cells consisted of ≈80% of authentic [^{3}H]Ach, was largely Ca^{2+}-dependent and tetrodotoxin sensitive, and hence represents an action potential-evoked, exocytotic release of acetylcholine. Using pairs of selective agonists and antagonists added before S_2, muscarinic autoreceptors, as well as inhibitory adenosine A1- and opioid μ-receptors, could be detected, whereas δ-opioid receptors were not found. Evoked [^{3}H] overflow from cultures grown for 1 week, although Ca^{2+} dependent and tetrodotoxin sensitive, was insensitive to the muscarinic agonist oxotremorine, whereas the effect of oxotremorine on cells grown for 3 weeks was even more pronounced than that in 2-week-old cultures. In conclusion, similar to observations on rat septal tissue *in vivo,* acetylcholine release from septal cholinergic neurones grown *in vitro* is inhibited via muscarinic, adenosine A_1 and μ-opioid receptors. This *in vitro* model may prove useful in the exploration of regulatory mechanisms underlying the expression of release modulating receptors on septal cholinergic neurones.

Keywords: adenosine A_1 receptors, development, electrically evoked release of acetylcholine, muscarine autoreceptors, opioid μ-receptors, septal cell cultures.

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Cholinergic innervation of the hippocampus originates mainly from the medial septum and the diagonal band of Broca (Amaral and Witter 1995; Jakab and Leranth 1995). The release of acetylcholine (Ach) in the target area of this projection, the hippocampus, has been shown to be modulated via muscarinic autoreceptors (Starke *et al*. 1989) and various heteroreceptors. For example, μ-opioid receptors (Jackisch *et al*. 1986; Lapchak *et al*. 1990; Jackisch 1991) and adenosine A1-receptors (Jackisch *et al*. 1984; Duner-Engstrom and Fredholm 1988; Cunha *et al*. 1994) have been found on cholinergic axon terminals in the hippocampus. In the cell body region of the septohippocampal projection, the medial septum and the diagonal band of Broca, we observed

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Abbreviations used: Ach, acetylcholine; AchE, acetylcholinesterase; ChAT, choline acetyltransferase; CPA, N^6-cyclopentyladenosine; DAGO (D-Ala^2, N-Me-Phe^4, $glycinol^5$)-enkephalin; DIV, days *in vitro*; DPCPX, 1,8-dipropyl-8-cyclopentylxanthine; DPDPE (D-Pen^2, D-Pen^5)-enkephalin; ED, embryonic day; GFAP, glial fibrillary acidic protein; hNT-3, human neurotrophin-3; Krebs–Henseleit, modified Krebs–Henseleit buffer; MAP_{II}, microtubule associated protein II; NGF, nerve growth factor.

that the evoked release of Ach was inhibited via muscarinic autoreceptors (Disko *et al*. 1998, 1999), as well as via μ- and δ-opioid receptors (Gazyakan *et al*. 2000a) and adenosine A1-receptors (unpublished observations).

Little is known about the perinatal expression and development of presynaptic receptors on cholinergic neurones of the CNS. Marchi *et al*. (1983) followed the development of muscarinic autoreceptors on synaptosomes of the rat neocortex, whereas DeVries *et al*. (1990) and Perez-Navarro *et al*. (1993) studied the ontogeny of various presynaptic receptors on cholinergic nerve terminals in the rat striatum. Using the technique of electrically evoked Ach release from brain slices, we recently reported on the postnatal development of presynaptic muscarinic and opioid receptors on cholinergic neurones in the hippocampus and the septum of the rat (Disko *et al*. 1998, 1999; Goldbach *et al*. 1998; Gazyakan *et al*. 2000b).

Although the time course of the postnatal appearance of presynaptic receptors could be established in the studies mentioned above, the signals and events regulating their expression remain to be clarified. Such regulatory mechanisms can, however, be studied more easily on neuronal systems *in vitro* (Héry *et al*. 1999). Therefore, the first aim of this study was to investigate the basic properties of a cell culture model (septal cholinergic neurones) in which electrically evoked Ach release and its modulation could be measured. Secondly, we wanted to find out which of the presynaptic receptors found on cholinergic cells *in vivo*, i.e. in the cell body or the target area of the septohippocampal pathway (see above), are also detectable *in vitro*, i.e. in cultures of septal cholinergic neurones.

Materials and methods

Materials

Chemicals and drugs were purchased from the following sources: [methyl-^{3}H]choline chloride (85 Ci/mmol) and [^{14}C]acetyl coenzyme A (51 mCi/mmol) from Amersham-Pharmacia (Braunschweig, Germany); ATP, N^6-cyclopentyladenosine (CPA), 1,8-dipropyl-8-cyclopentylxanthine (DPCPX), methiotepine mesylate (metitepine), naloxone hydrochloride and poly D-lysine from RBI (Biotrend) (Köln, Germany); acetylcholinesterase (AchE; EC 3.1.1.7) type VI-S from electric eel, atropine sulfate, choline oxidase (EC 1.1.3.17) from *Alcaligenes* species, hemicholinium-3, oxotremorine sesquifumarate, paraoxon and tetrodotoxin from Sigma-Aldrich (München, Germany); (D-Ala^2, N-Me-Phe^4, glycinol^5)-enkephalin (DAGO), (D-Pen^2, D-Pen^5)-enkephalin (DPDPE) from Bachem (Heidelberg, Germany); and sodium tetraphenyl borate and butyronitrile from Merck (Darmstadt, Germany). Sources for products used for cell cultivation and immunocytochemistry are indicated below.

Preparation of septal cell cultures

Pregnant Wistar rats on embryonic day 17 (ED 17) were anaesthetized with sodium pentobarbital, the embryos were removed and their brains dissected under a stereomicroscope according to Svendsen (1995). Tissue pieces containing the septal region were collected in ice-cold phosphate-buffered saline (Biochrom KG, Berlin, Germany) and dissociated by incubation for 20 min at 37°C in 1 mL of trypsin-EDTA solution (0.5%; Sigma-Aldrich). Trypsinization was stopped by the addition of 500 μL Viable AC-2 medium (Pan Systems; Nürnberg, Germany). Cells were subsequently dissociated by gentle trituration using a fire-polished Pasteur pipette. The suspension was centrifuged at 200 *g* for 5 min and the pellet resuspended in 1 mL growth medium (see below). This suspension was re-centrifuged (200 *g*, 5 min) and resuspended in the final volume (125 μL for cells obtained from one septum) of growth medium (see below) required to yield ≈650 000 cells/cm^2 (counted before plating of the suspension). Cells were plated onto 90-well plates containing 1 circular (5 mm diameter) glass coverslip precoated with poly D-lysine (Sigma-Aldrich) per well. In some experiments 10 μL of a more concentrated cell suspension (17.5 μL for cells obtained from one septum) were pipetted directly onto each of 12 glass coverslips on the bottom of Petri dishes (35 mm diameter; Falcon/Schubert; München, Germany). After incubation of these dishes for 2 h at 37°C in a humidified 95% air/5% CO_2 atmosphere, 1.2 mL of growth medium was carefully added to the culture dish.

Cells were cultured at 37°C in a humidified 95% air/5% CO_2 atmosphere for 7, 14 or 21 days [day *in vitro* (DIV) 7, 14 or 21) in Dulbecco's modified Eagle's medium (Nut. Mix. F-12 with Glutamax-1, GibcoBRL, Life Technologies, Eggenstein, Germany) containing 10% Viable AC-2, a mixture of insulin, transferrin and sodium selenite (5 μg/mL, 5 μg/mL and 5 ng/mL; Sigma-Aldrich), a mixture of penicillin and streptomycin (50 U/mL and 50 μg/mL; GibcoBRL), as well as mouse nerve growth factor (mNGF; 10 ng/mL) and human neurotrophin-3 (hNT3; 50 ng/mL; Alomone/ICS, München, Germany). The growth medium was changed every 3–4 days.

Immunostaining of cells

Acetyl-CoA : choline O-acetyltransferase (EC 2.3.1.6), choline acetyltransferase

Cultured cells were washed with 0.1 mol/L sodium phosphate buffer (pH 7.35) and fixed with sodium phosphate buffer containing 4% paraformaldehyde and 0.2% picric acid. Following fixation, cultures were washed for 30–60 min and then incubated for 2 days at 4°C with choline acetyltransferase (ChAT) rat monoclonal antibody (1 μg/mL; Boehringer, Mannheim, Germany), 0.1% Triton X-100, 0.1% sodium azide and 1% rabbit serum in sodium phosphate buffer. Cultures were washed with sodium phosphate buffer (60 min) and incubated with biotinylated antirat antibody (1 : 230; Vector Laboratories; Burlingham, USA) in sodium phosphate buffer for ≈12 h. The cells were subsequently washed and incubated with an avidin–biotin conjugate of peroxidase (Vectastain, Vector Laboratories) for 2 h and then washed again. Peroxidase was visualized using diaminobenzidin (0.7 mg/mL; Sigma-Aldrich) and hydrogen peroxide (0.1%).

Glial fibrillary acidic protein

The glial marker was visualized according to Manthorpe *et al*. (1979). Cultures were fixed with 4% paraformaldehyde in sodium phosphate buffer (30 min) then washed and treated with 4% normal

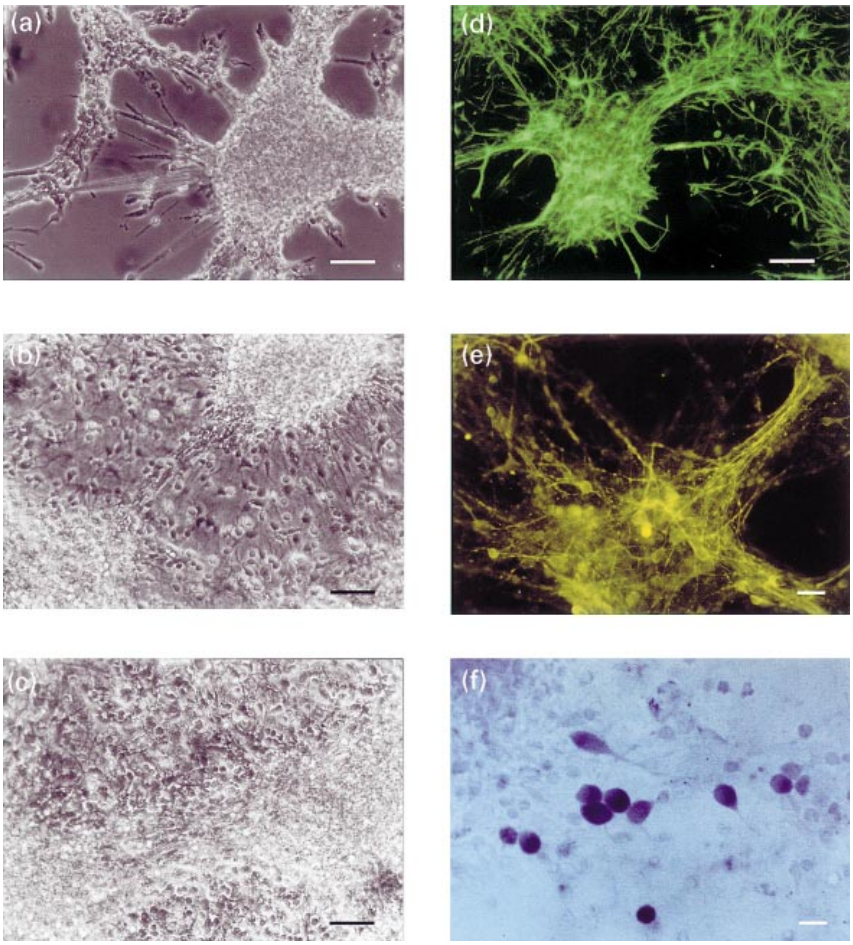

Fig. 1 Microscopic aspects of septal cell cultures. Phase contrast microscopic aspect of septal cells cultured for (a) 7, (b) 14 or (c) 21 days *in vitro*. Immunostaining of septal cells (DIV 14) for the (d) glial marker glial fibrillary acidic protein (GFAP), (e) the neuronal marker microtubule-associated protein II (MAP_{II}) or (f) the cholinergic marker choline acetyltransferase (ChAT). The bar in a–d represents 100 μm, that in (e) and (f) represents 20 μm.

goat serum (Vector Laboratories) and 0.1% Triton X-100. Thereafter the cells were incubated for 12 h at 4°C with glial fibrillary acidic protein polyclonal rabbit antibody (1 : 600; Boehringer) and then stained by the addition of Cy2-conjugated goat antirabbit antibody (1 : 600; Jackson Immunoresearch Laboratories; West Grove, USA) for 2 h. Washes in between steps were carried out with phosphate-buffered saline.

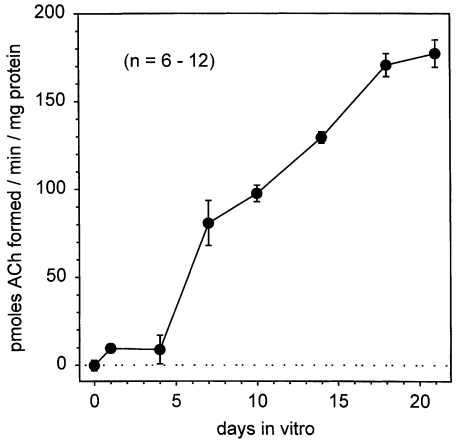

Fig. 2 Time course of the development of choline acetyltransferase (ChAT) activity in septal cell cultures during growth ($n = 6–12$ single determinations from two independent cell cultures).

Microtubule-associated protein

To visualize neuronal microtubule-associated protein II (MAP_{II}) a mouse monoclonal antibody was used (1 : 200; Sigma-Aldrich). The cultures were fixed and washed as described for GFAP immunostaining. As a second antibody, goat antimouse IgG conjugated with Cy3 (1 : 600; Rockland, Gilbertsville, USA) was used and incubated for 2 h. The stained cultures were washed with water and mounted in moviol (Hoechst, Frankfurt, Germany).

Determination of ChAT activity

Cultured septal cells (from four glass coverslips per sample), grown for various DIV were carefully scraped off, homogenized in 400 μL 0.32 mole/L sucrose (in 2.5 mmol/L HEPES, pH 7.4) and stored at −80°C until determination. The ChAT activity in these samples was measured according to Fonnum (1975) as described in detail previously (Cassel *et al*. 1993). The protein content of the cell homogenates was assessed according to Lowry *et al*. (1951).

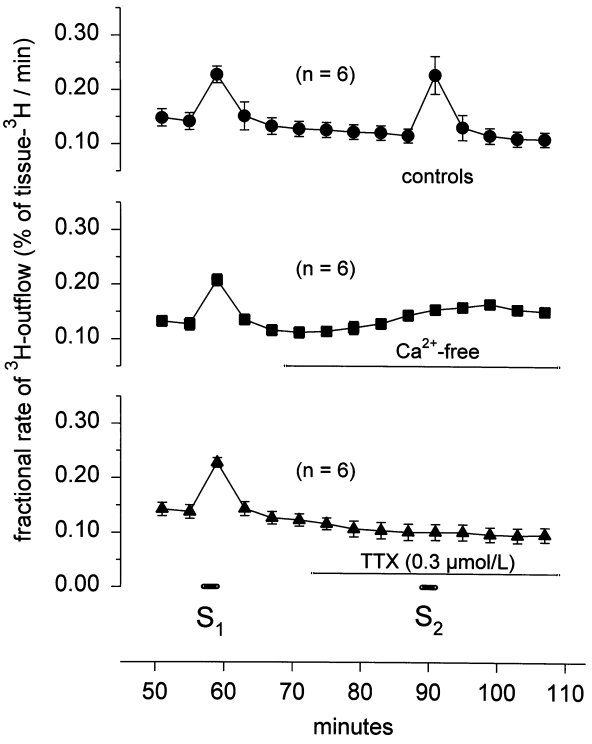

Fig. 3 Time course of [^{3}H]outflow from septal cell cultures (DIV 14): effects of Ca^{2+}-free medium and tetrodotoxin (TTX). Septal cells cultured on glass coverslips for 14 days were pre-incubated with [^{3}H]choline and then superfused continuously with medium containing hemicholinium-3 (10 µmol/L). During superfusion they were stimulated electrically twice after 57 min (S_1) and 89 (S_2) min of superfusion, as indicated by the black bars. Calcium was omitted from, or tetrodotoxin (0.3 µmol/L) added to, the medium from 20 or 16 min, respectively, before S_2 onwards. For the amount of [^{3}H]overflow evoked by S_1 and the extent of drug effects on the S_2/S_1 ratio see Fig. 4.

Electrically evoked release of acetylcholine

Twenty-four circular coverslips with septal cells (cell culture discs), grown for 7, 14 or 21 days (DIV 7, 14 or 21), were transferred to a 35-mm Petri dish filled with cooled, modified Krebs–Henseleit buffer [composition in mmol/L: NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11; ascorbic acid, 0.57; Na_2EDTA, 0.03; saturated with carbogen (95% O_2, 5% CO_2), pH adjusted to 7.4] and washed carefully with this medium two or three times. The cell culture discs were subsequently incubated for 30 min at 37°C in 2 mL Krebs–Henseleit buffer containing [^{3}H]choline (0.1 µmol/L) under an atmosphere of carbogen. Following incubation they were washed with prewarmed and gassed Krebs–Henseleit buffer, transferred to 12 superfusion chambers (two cell culture discs, back to back, per chamber) and superfused continuously with Krebs–Henseleit buffer (25°C, gassed with carbogen) at a rate of 0.6 mL/min in the presence of hemicholinium-3 (10 µmol/L). The collection of 4-min fractions was started after 45 min of superfusion; electrical field stimulation for 2 min (360 rectangular pulses, 3 Hz, 0.5 ms, 90–100 mA, 8.5 V/chamber) was applied after 57 min (S_1) and 89 min (S_2) of superfusion. Drugs to be tested were added to the superfusion medium from 20 min (Ca^{2+}-free medium) or 16 min (all other drugs) before S_2 onwards. At the end of the experiment (after 107 min of superfusion), the radioactivity of the superfusate samples and the cell culture discs (cells dissolved in 250 µL Soluene 350; Packard) was determined using liquid scintillation counting.

Determination of the [^{3}H]Ach content in the basal [^{3}H]efflux and the electrically evoked [^{3}H]overflow of the cultured cells was performed in separate experiments. Cultured cells (DIV 14) were pre-incubated with [^{3}H]choline as above for 30 min. Subsequently, 50 µM paraoxon (to irreversibly block AchE in the cultures) was added and the cells were incubated for a further 15 min. Finally, they were washed, superfused (in the absence of paraoxon, but the presence of hemicholinium-3, 10 µmol/L) and stimulated electrically once only (S_1) as described above. Aliquots (800 µL) of the superfusate fractions were counted directly to determine total [^{3}H]outflow (value A). Two further aliquots (800 µL each) of fractions 53–57 min (b_1) and 57–61 (S_1) min of superfusion were incubated (20 min at 37°C) with 80 µL of a mixture containing either (value B) choline oxidase (1.6 U in 500 mmol/L Tris, pH 8.0) or (value C) choline oxidase (1.6 U) plus AchE (3.2 U in 500 mmol/L Tris pH 8.0). At the end of the incubation, 500 µL of sodium tetraphenyl borate in butyronitrile (10 mg/mL) was added, and the mixture was shaken vigorously for 2 min and centrifuged for 5 min (22 800 *g*). Three hundred microlitres of the upper (organic) phase were taken for liquid scintillation counting. From the differences between value B ([^{3}H]choline removed by choline oxidase: only [^{3}H]Ach extracted into the organic phase) and value C (both [^{3}H]choline and [^{3}H]Ach enzymatically removed: the organic phase contains only nonspecifically extracted [^{3}H]) the amount of [^{3}H]Ach in the superfusate samples was calculated as a percentage of the total radioactivity (value A) in the superfusate fraction.

Calculations and statistics

The fractional rate of tritium outflow (% cell tritium per min) was calculated as: (pmoles tritium outflow per 4 min) × 100/4 × (pmoles tritium in the 'cell culture discs' at the start of the respective 4-min period). The stimulation-evoked overflow of tritium was calculated by subtracting the basal outflow; the latter was assumed to decline linearly from 4 min before to the 4-min period 12–16 min after the onset of the stimulation. The evoked overflow was expressed as the per cent of the tritium content of the cell culture discs at the onset of the respective stimulation period. The effects of drugs added before S_2 were determined as the ratio of the overflow evoked by the corresponding stimulation periods (S_2/S_1) and compared with the appropriate control ratios (same age of culture; no drug addition before S_2). The effects of drugs added before S_2 on the basal outflow of [^{3}H] were determined as the ratio (b_2/b_1) of the fractional rates of [^{3}H]outflow of the fractions preceding the two stimulation periods and compared with the appropriate control ratios (same age of culture; no drug addition before S_2). Estimation of the IC_{50} and the maximal inhibitory effect of oxotremorine at the muscarinic autoreceptor were performed using non-linear regression analysis (Feuerstein *et al.* 1993) of the individual S_2/S_1 ratios, which are shown as means ± SEM. The question of whether endogenous Ach competes with oxotremorine

at the autoreceptor was studied using another linear regression analysis model (Feuerstein *et al*. 1987, 1992).

Significance of differences was tested using ANOVA followed by Bonferroni's test or nonparametric ANOVA (Kruskal–Wallis test) followed by Dunn's test. All data are shown as means ± SEM; *n* is the total number of cell culture discs in at least four independent superfusion experiments (i.e. from four independent cell cultures).

Results

Morphological aspect of septal cell cultures

Dissociated septal cells (from ED 17) grew well in medium supplemented with mouse NGF and human NT-3 on circular poly D-lysine-coated glass coverslips. By 18 h after seeding, many cells had started to produce extensions from their cell bodies (not shown). After 7 days *in vitro* (DIV 7), multiple aggregates of cells could be observed on the coverslips, aggregates which were extensively linked to each other by multiple neurites (Fig. 1a). During further growth, the density of this network and the number of cells per aggregate increased, so that the circular glass discs were covered almost completely by a multilayer cellular network at DIV 14 (Fig. 1b); over the next week (DIV 21) the density of this network appeared to increase further (Fig. 1c).

As evident from immunocytochemical staining of the cells around DIV 14 (i.e. the time when most of the functional experiments were performed), these septal cultures consisted of a network of neuronal and non-neuronal cells. For example, using an antibody for GFAP (Fig. 1d), the presence of glial cells in the aggregates was evident, whereas staining for the neuronal marker protein MAP_{II} (Fig. 1e) revealed a large number of neuronal cells in the network, some of which contained the GABAergic marker glutamate decarboxylase (not shown). Finally, as revealed by staining with ChAT antibodies (Fig. 1f), a significant number of cholinergic cells was detectable in or between the cell clusters. Because of the high density of the cellular network, however, it was not possible to quantify the number of cholinergic cells compared with other neuronal or non-neuronal cells. Nevertheless, as shown in Fig. 2, the amount of enzymatically determined ChAT activity increased significantly during growth of septal cell cultures *in vitro*.

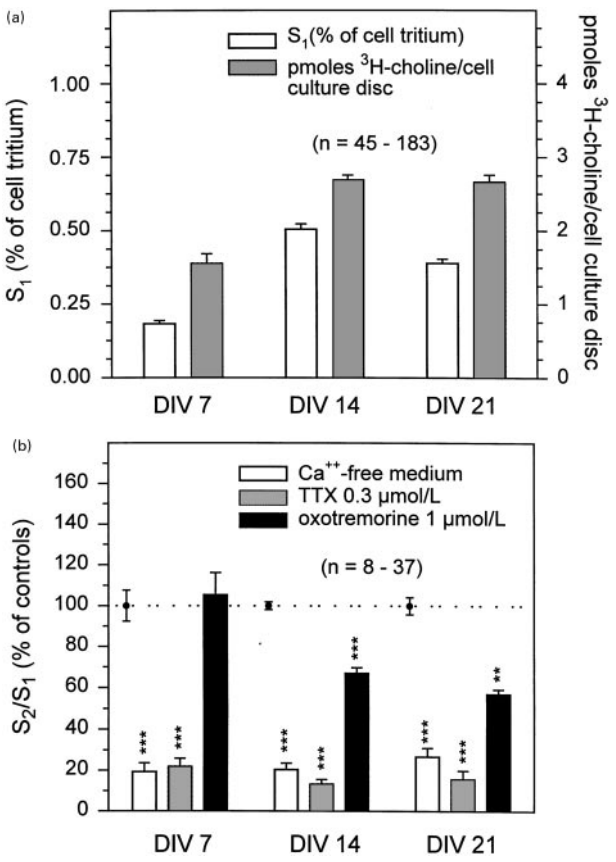

Fig. 4 Basic properties of the transmitter release model. Summary of experiments on septal cell cultures (DIV 7, 14 and 21) pre-incubated, superfused and electrically stimulated as shown in Fig. 3. (a) Accumulation of [^{3}H]choline and electrically evoked [^{3}H]overflow at S_1. Significance of differences between [^{3}H]choline accumulation values (pmoles [^{3}H]choline per cell culture disc): DIV 7 versus DIV 14: $p < 0.001$; DIV 14 versus DIV 21: n.s.; significance of differences in between S_1 values: DIV 7 versus DIV 14: $p < 0.001$; DIV 14 versus DIV 21: $p < 0.01$. (b) Effects of Ca^{2+}-free medium, tetrodotoxin (0.3 μmol/L) and oxotremorine (1 μmol/L) on the electrically evoked [^{3}H]overflow at S_2. Effects are shown as the ratio S_2/S_1 as a percentage of the corresponding drug-free controls (symbols with error bars at the dotted 100% reference line). Significance versus corresponding controls: $^{***}p < 0.001$; $^{**}p < 0.01$; significance in between drug effects at different cultivation delays: Ca^{2+}-free medium and tetrodotoxin: DIV 7 versus DIV 14: n.s.; DIV 14 versus DIV 21: n.s.; oxotremorine: DIV 7 versus DIV 14: $p < 0.01$; DIV 14 versus DIV 21: $p < 0.05$.

Electrically evoked release of acetylcholine

General properties and modulation via muscarinic autoreceptors

Pre-incubation of septal cells (DIV 14) with [^{3}H]choline, followed by continuous superfusion (in the presence of hemicholinium-3) and electrical field stimulation, led to the pattern of [^{3}H]outflow shown in Fig. 3. It is evident that electrical field stimulation induced an [^{3}H]outflow that was significantly higher than the basal [^{3}H]outflow. This 'stimulation-evoked [^{3}H]overflow' was Ca^{2+} dependent and sensitive to the presence of tetrodotoxin (see below). In separate experiments on septal cells cultured for 2 weeks (DIV 14) we also determined the amount of authentic [^{3}H]Ach in the tritiated outflow, both in the fraction preceding S_1 (fraction 2 in Fig. 3: basal [^{3}H]outflow) and in the fraction during which electrical field stimulation was

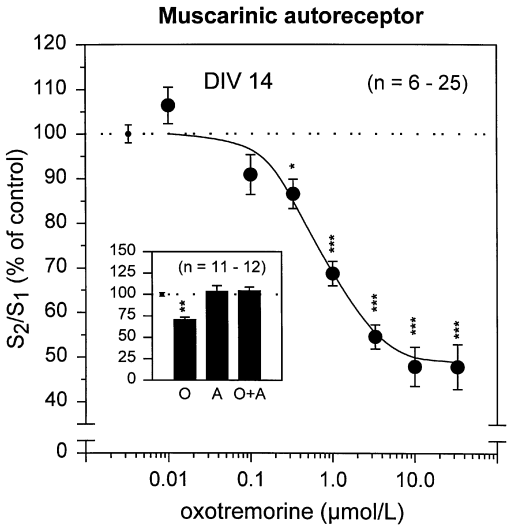

Fig. 5 Effects of oxotremorine on the electrically evoked [^{3}H]overflow from septal cell cultures pre-incubated with [^{3}H]choline. Septal cells (DIV 14) were pre-incubated, superfused and stimulated as shown in Fig. 3. Oxotremorine was added to the medium in concentrations shown on the abscissa from 16 min before S_2 onwards. Its effects are expressed as a percentage of the corresponding drug free controls (small symbol with error bars at the dotted 100% reference line). (Inset) Effects of oxotremorine (O, 1 µmol/L), atropine (A, 1 µmol/L) and a combination of both (O + A, 1 µmol/L each) as expressed in percentage of the corresponding controls (small symbol with error bars at the dotted 100% reference line). Significance of differences versus drug-free controls: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

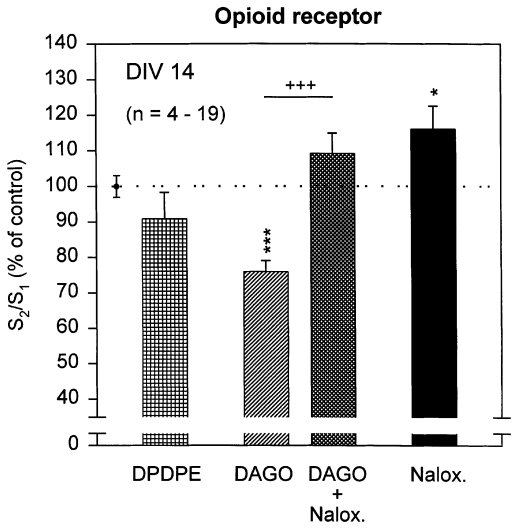

Fig. 6 Effects of the δ-opioid receptor agonist (D-Pen^2, D-Pen^5)-enkephalin (DPDPE; 1 µmol/L), the µ-opioid receptor agonist D-Ala^2, N-Me-Phe^4, glycinol^5)-enkephalin (DAGO; 1 µmol/L) and the preferential µ-receptor antagonist naloxone (0.1 µmol/L) on the electrically evoked [^{3}H]overflow from septal cell cultures pre-incubated with [^{3}H]choline. Septal cells (DIV 14) were pre-incubated, superfused and stimulated as shown in Fig. 3. Opioid receptor agonists and antagonists were added to the medium from 16 min before S_2 onwards. Their effects are expressed as the percentage of the corresponding drug free controls (small symbol with error bars at the dotted 100% reference line). Significance of differences versus drug-free controls: $^{***}p < 0.001$, $^{*}p < 0.05$, or versus DAGO 1 µmol/L, given alone: $^{+++}p < 0.001$.

performed (fraction 3 in Fig. 3; from this value the 'evoked [^{3}H]overflow' was calculated by subtraction of fraction 2). It was found (values are given as a percentage of the total radioactivity in the corresponding fractions) that the basal [^{3}H]outflow consisted of 15.8 ± 1.2% [^{3}H]Ach, whereas the evoked [^{3}H]overflow consisted of 78.0 ± 4.6% of [^{3}H]Ach ($n = 14$, each; two independent experiments on two different septal cultures).

Figure 4 summarizes experiments on septal cells cultivated for 7, 14 or 21 days, pre-incubated with [^{3}H]choline, superfused and electrically stimulated as shown in Fig. 3. The accumulation of [^{3}H]choline (pmoles ^{3}H-choline/cell culture disc) increased significantly ($p < 0.001$) between 1- and 2-week-old cultures, but remained unchanged following one additional week of culture (Fig. 4a). The evoked overflow of [^{3}H] at S_1 was significantly lower at DIV 7 and 21 than at DIV 14 both in relative terms (i.e. S_1 as a percentage of cell-^{3}H, see Fig. 4a legend) and in absolute terms [i.e. S_1 in nCi: DIV 7 ($n = 45$), 0.242 ± 0.028, $p < 0.001$ versus DIV 14 ($n = 183$), 1.167 ± 0.048, $p < 0.001$ versus DIV 21 ($n = 94$), 0.839 ± 0.049]. In contrast, the basal outflow rate of [^{3}H] in the fraction preceding S_1 (i.e. 53–57 min of superfusion, b_1) did not differ in septal cell cultures grown *in vitro* for 1, 2 or 3 weeks [b_1 as a percentage of cell-^{3}H/min: DIV 7, 0.128 ± 0.010 ($n = 45$); DIV 14, 0.128 ± 0.003 ($n = 183$); DIV 21, 0.135 ± 0.004 ($n = 94$)].

Figure 4(b) shows the effects of Ca^{2+}-free medium, tetrodotoxin (0.3 µmol/L) and the muscarinic autoreceptor agonist oxotremorine (1 µmol/L) on the electrically evoked overflow of [^{3}H] from septal cells. It is evident that the evoked overflow of [^{3}H] was significantly dependent on the presence of extracellular Ca^{2+} (open bars) and strongly reduced in the presence of tetrodotoxin (0.3 mmol/L; solid bars) at all developmental stages of the cultures. It should be mentioned, however, that omission of Ca^{2+} (see also Fig. 3) also slightly increased the basal outflow of [^{3}H] in experiments on 2- and 3-week-old cultures [b_2/b_1 ratios as a percentage of corresponding controls: DIV 7, 107 ± 5 ($n = 12$; n.s.); DIV 14, 123 ± 5 ($n = 30$; $p < 0.001$); DIV 21, 119 ± 3 ($n = 28$; $p < 0.01$)].

The muscarinic agonist oxotremorine (1 µmol/L; black columns in Fig. 4b) did not inhibit the evoked [^{3}H]overflow at DIV 7, whereas at DIV 14 a significant inhibitory effect (≈30%) was observed, which further increased significantly

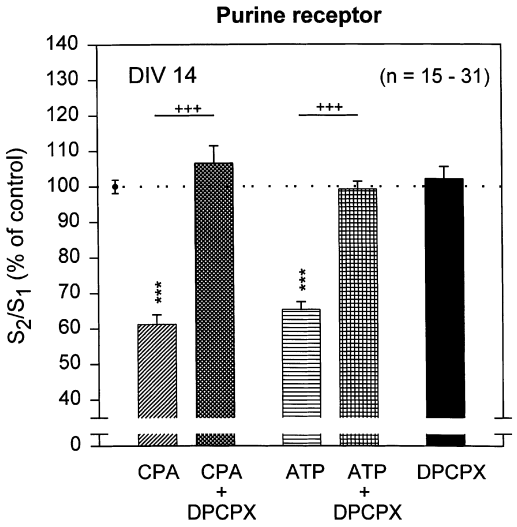

Fig. 7 Effects of the purine receptor agonists N^6-cyclopentyladenosine (CPA; 1 μmol/L) and ATP (100 μmol/L), and of the selective A1-receptor antagonist 1,8-dipropyl-8-cyclopentylxanthine (DPCPX; 0.1 μmol/L) on the electrically evoked [^{3}H]overflow from septal cell cultures pre-incubated with [^{3}H]choline. Septal cells (DIV 14) were pre-incubated, superfused and stimulated as shown in Fig. 3. Purine receptor agonists and antagonists were added to the medium from 16 min before S_2 onwards. Their effects are expressed as a percentage of the corresponding drug free controls (small symbol with error bars at the dotted 100% reference line). Significance of differences versus drug-free controls: $^{***}p < 0.001$, or versus CPA (1 μmol/L), or ATP (100 μmol/L) given alone: $^{+++}p < 0.001$.

($p < 0.05$) following an additional 1 week's culture (at DIV 21, ≈42% inhibition). The inhibitory effect of oxotremorine was studied in more detail on septal cells cultured for 2 weeks and a sigmoid concentration–response relationship of oxotremorine was observed (Fig. 5). Non-linear regression analysis of the data shown in Fig. 5 yielded an IC_{50} of 0.86 μmol/L (95% confidence interval: 0.57; 1.40 μmol/L) and a maximal inhibitory effect of oxotremorine (as percentage of controls) of 54% (95% confidence interval: 48; 64); the slope parameter c of the fitted curve was found to be around unity [c = 1.10 (95% confidence interval: 0.73, 1.78)]. Non-linear regression analysis of the same data using another model (Feuerstein *et al.* 1987, 1992), which permits estimation of the concentration of endogenous Ach at the autoreceptor during the first stimulation, did not reveal any significant inhibitory tone of the endogenous agonist at the autoreceptor. Figure 5 (insert) shows that the effect of oxotremorine (1 μmol/L) was abolished in the presence of atropine (1 μmol/L), which when given alone did not affect the evoked [^{3}H]overflow. It should also be noted that physostigmine (1 μmol/L), an inhibitor of AchE (EC 3.1.1.7), did not significantly diminish the evoked [^{3}H]overflow (data not shown).

Modulation via heteroreceptors

The possible presence of further receptors modulating Ach release from septal cells cultured *in vitro* was studied only on 2-week-old cultures (DIV 14) pre-incubated with [^{3}H]choline. Figure 6 shows the effects of opioid receptor agonists and antagonists on the electrically evoked [^{3}H]overflow from these cultures. Whereas the preferential μ-opioid receptor agonist DAGO (1 μmol/L) significantly inhibited the evoked [^{3}H]overflow, the preferential δ-opioid receptor agonist DPDPE (1 μmol/L) showed only a slight inhibitory tendency. The opioid receptor antagonist naloxone (0.1 μmol/L) antagonized the effect of DAGO and, interestingly, enhanced the evoked [^{3}H]overflow when given alone. None of the drugs shown in Fig. 6 affected the basal outflow of [^{3}H].

The effects of drugs binding to purinergic receptors on the evoked [^{3}H]overflow from these cells are shown in Fig. 7. Both the selective P_1-receptor agonist CPA (1 μmol/L) and the unselective P_2-agonist ATP (100 μmol/L) significantly inhibited the evoked [^{3}H]overflow by ≈35–40%. Interestingly, however, the effect of not only the P_1-agonist CPA, but also that of the P_2-agonist ATP, was antagonized completely in the presence of the selective P_1 antagonist DPCPX (0.1 μmol/L), which given alone did not affect the evoked [^{3}H]overflow. None of the drugs shown in Fig. 7 affected the basal outflow of [^{3}H].

Discussion

It was the aim of this study to establish a model for the electrically evoked release of Ach from cholinergic neurones cultured *in vitro*. For this purpose, septal cells from the embryonic rat brain (ED 17) were grown in medium supplemented with NGF and hNT-3 (Hartikka and Hefti 1988; Nonner *et al.* 1996; Murata *et al.* 1998). The tentative morphological characterization (Fig. 1) of these septal cultures shows that they represent a mixture of non-neuronal and neuronal cells. A more detailed phenotypic characterization of the septal cell cultures was beyond the scope of this study.

Several lines of evidence support the presence of cholinergic cells in these cultures: firstly, the staining of some of the cells using an antibody for ChAT (Fig. 1), secondly, the increase in ChAT activity during cultivation (Fig. 2), and thirdly, the observation that electrical field stimulation of these cultures (following pre-incubation with [^{3}H]choline) elicits an overflow of tritium (Fig. 3), which consisted of ≈80% of authentic [^{3}H]Ach. It has been suggested previously (Molenaar *et al.* 1973; Richardson and Szerb 1974), that evoked [^{3}H]overflow from cells or tissues pre-incubated with [^{3}H]choline reflects the release of [^{3}H]Ach. Our present data also show that this evoked release of Ach was already action potential induced

(tetrodotoxin sensitive) and exocytotic (largely Ca^{2+} dependent) by DIV 7. To our knowledge, this is the first time that the electrically evoked release of Ach from cultured septal cells in a continuous superfusion system has been described.

During growth of the septal cultures tissue accumulation of [^{3}H]choline increased up to a maximum at DIV 14, whereas the electrically evoked [^{3}H]overflow increased from DIV 7 to 14 but decreased from DIV 14 to 21 (Fig. 4a). These observations are difficult to explain because changes in high-affinity [^{3}H]choline uptake and [^{3}H]Ach synthesis during development have to be taken into account. Moreover, [^{3}H]choline may also be used by noncholinergic (and non-neuronal) cells for the synthesis of membrane lipids. Therefore, it remains to be established whether the observations depicted in Fig. 4(a) really reflect changes in density or function of cholinergic neurones.

It should be noted, however, that a significant increase in the inhibitory effects of the muscarinic agonist oxotremorine (at its IC_{50} concentration) was observed from DIV 7 to 21, suggesting an increase in the expression of the muscarinic autoreceptors during development. Although this finding is only preliminary and has to be confirmed by further studies, it supports the time course of the development of muscarinic autoreceptors observed *in vivo* in both the hippocampus (Goldbach *et al*. 1998) and the septum of rats (Disko *et al*. 1998, 1999). Furthermore, it underlines the possible usefulness of the cell culture model to explore regulatory mechanisms in the expression of presynaptic receptors in septal cholinergic neurones.

A more detailed investigation of the effect of oxotremorine (Fig. 5) revealed that it was atropine sensitive and hence due to the activation of muscarinic receptors. Atropine alone did not enhance the evoked Ach release, suggesting that the concentration of endogenous Ach around the autoreceptor was ≈ 0, a suggestion also supported by estimations of this Ach concentration using non-linear regression analysis of the oxotremorine concentration–response curve (see Results). Most probably, newly released Ach is rapidly washed away by the flow of the superfusate, or metabolized by AchE. The latter possibility seems, however, to be less probable because the AchE inhibitor physostigmine added before S_2 did not inhibit the evoked release of Ach.

The IC_{50} value of oxotremorine on the evoked release of Ach from cultured cholinergic (septal) neurones was estimated using non-linear regression analysis (Feuerstein *et al*. 1993). The slope parameter c of this analysis was around unity suggesting a direct proportionality between receptor occupation and response (Agneter *et al*. 1997; Feuerstein and Limberger 1999). Because acetylcholine and oxotremorine are approximately equipotent at muscarinic receptors (Cho *et al*. 1962), it can be assumed (Feuerstein *et al*. 1992) that the IC_{50} value for oxotremorine also reflects its K_D value (i.e. pK_D, 6.07; 95% confidence interval, 6.24, 5.85) and that of Ach itself at the autoreceptor. This value is very similar to the pK_D value for oxotremorine at the muscarinic autoreceptor in rat brain cortex slices (Albrecht *et al*. 1999), suggesting that the subtype of muscarinic autoreceptor in septal cholinergic neurones cultured *in vitro* is similar to that observed in the rat brain cortex *in vivo*.

In addition to muscarinic autoreceptors, several inhibitory heteroreceptors, for example μ- and δ-opioid receptors, as well as adenosine A1-receptors, have also been described on cholinergic axon terminals in the rat brain (see Introduction). In agreement with these observations *in vivo*, Ach release from cultured septal cells was also inhibited following activation of opioid- and adenosine receptors. However, using the subtype-selective μ-agonist DAGO (Kosterlitz and Paterson 1981) and the δ-agonist DPDPE (Cotton *et al*. 1985), only μ- and no δ-opioid receptors could be detected at DIV 14 (Fig. 6). This finding may be related to the observation that *in vivo* δ-opioid receptors also appear later in ontogenetic development than μ-opioid receptors (Kornblum *et al*. 1987; DeVries *et al*. 1990; Gazyakan *et al*. 2000b). Interestingly, the preferential μ-opioid receptor antagonist naloxone not only antagonized the effect of DAGO, but also exhibited significant facilitatory effects when given alone (Fig. 6). This effect may be due to the presence of endogenous opioid peptides in the cultures that tonically activate μ-opioid receptors on cholinergic neurones. Therefore, it seems possible that opioid peptide-producing neurones occurring in the septal area *in vivo* (Augood *et al*. 1997) also develop in cell cultures from the embryonic septum.

Because not only the inhibitory effect of the A_1 adenosine receptor agonist CPA (1 μmol/L), but also that of ATP (100 μmol/L) was antagonized by a low concentration (0.1 μmol/L) of the A1-receptor antagonist DPCPX, only inhibitory A1-receptors, and not P_2-receptors, seem to modulate Ach release in cultured septal cells. The inhibitory effect of ATP may be mediated either directly via the A1-receptor, or subsequent to a breakdown to adenosine by an ecto-ATPase associated with cholinergic nerve terminals (Cunha and Sebastiao 1992). The preferential A1-receptor antagonist given alone did not enhance the evoked release of Ach, indicating that, in contrast to K^+-evoked Ach release from cultured amacrine cells (Santos *et al*. 1998), endogenous adenosine does not tonically inhibit the release of Ach in septal cell cultures.

In conclusion, we have shown that the action-potential evoked exocytotic release of Ach can be induced by electrical field stimulation of rat septal cells in culture. In 14-day-old cultures this evoked release of Ach is modulated via muscarinic autoreceptors, μ-opioid and adenosine A1-receptors. This *in vitro* model may prove useful in the exploration of regulatory mechanisms underlying the

expression of release modulating receptors on septal cholinergic neurones.

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