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Depolarization of human neuroblastoma cells as a result of muscarinic receptor-induced rise in cytosolic Ca^{2+}

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The role of intracellular free Ca^{2+} in muscarinic-receptor linked depolarization of SH-SY5Y neuroblastoma cells has been determined by using the bisoxonol membrane potential probe $DiBaC_4$-(3) and intracellular Ca^{2+} indicator fura-2 respectively. Carbachol and the Ca^{2+} ionophore, ionomycin, at concentrations which caused similar rises in intracellular Ca^{2+} increased the bisoxonol fluorescence (depolarization) to the same extent. The membrane potential responses, but not the changes in intracellular Ca^{2+}, were dependent on extracellular Na^+. Ionomycin depletion of intracellular Ca^{2+} with EGTA and ionomycin or loading the cells with a Ca^{2+} buffer, BAPTA, reduced the carbachol-induced depolarization. The results suggest that a rise in intracellular Ca^{2+} may cause depolarization through an increase in the Na^+ permeability.

Muscarinic receptor; Depolarization; Carbachol; Fura-2; Bisoxonol; Ca^{2+}; Na^+

1. INTRODUCTION

Activation of receptors linked to phosphoinositol phospholipid breakdown causes complex changes in the electrical properties of neuroblastoma cells [1–4]. A transient initial hyperpolarization in response to bradykinin has been observed [1,2,4] followed by a depolarization phase. The hyperpolarization may be mimicked by microinjection of Ca^{2+} [1,4] while depolarization is obtained upon microinjection of 1,3,4 IP_3 [4]. Furthermore Ca^{2+} induces the activation of nonselective cation channels [5] in N1E115 murine neuroblastoma cells as well as an inward depolarizing current [1,3] in NG108-15 neuroblastoma/glioma hybrid cells. Apart from these changes depolarization due to activation of protein kinase C has also been observed in the NG108-15 hybrid cells. Muscarinic receptor-linked depolarization, $^{86}Rb^+$ efflux and Ca^{2+} mobilization has been seen in a ganglion cell derived human neuroblastoma cell line SH-SY5Y [6,7]. Depolarization upon activation of muscarinic receptors is generally thought to be due to the blocking of K^+ channels, the so called A-current [8]. In SH-SY5Y cells the A-current is observed with high frequency only after induction of differentiation with the phorbol ester TPA [9]. Thus it might be possible in these cells to observe changes in membrane potential as a result of other mechanisms than blocking of K^+-channels.

The aim of this study was to examine the role of Ca^{2+} in muscarinic receptor-linked depolarization in SH-SY5Y cells by measuring the membrane potential using the bisoxonol dye, $DiBaC_4$-(3) and Ca^{2+} using the fura-2 technique.

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Abbreviations: BAPTA/AM, 1,2-bis(*o*-aminophenoxy)-ethane *N,N,N',N'*-tetraacetic acid acetoxymethylester; fura-2/AM, fura-2-acetoxymethylester; H-EDTA, *N*-hydroxyethylenediamine triacetic acid; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

Carbachol and TPA were purchased from Sigma Chemicals Co. (St. Louis, MO). BAPTA/AM, fura-2/AM and $DiBaC_4$-(3) were obtained from Molecular Probes Inc. (Junction Ci-

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ty, USA). Ionomycin was from Calbiochem (Behring Diagnostics, La Jolla, CA). All the other reagents were commercial products of the highest purity available.

SH-SY5Y neuroblastoma cells [10] were obtained through Dr S. Påhlman (Department of Pathology, University of Uppsala, Sweden). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; KC Biologicals), supplemented with 7% fetal calf serum (Gibco, England) and 50 μg/ml gentamicin (Sigma Chemicals Co.) at 37°C in 5% CO_2 in an air ventilated humidified incubator. The cells used in the experiments were cultured to confluency, harvested using EDTA (0.02% in PBS) and washed twice in PBS. Subsequently they were seeded out at one third of the initial density in 800 ml culture flasks for fluorescence measurements. Experiments were carried out after 72 h of culture.

The cells were detached with EDTA and loaded with fura-2 in the DMEM medium by addition of 2 μM fura-2/AM and incubation with constant agitation for 20 min at 37°C. Thereafter the cells were spun down at 500 × g for 10 min and washed once by centrifugation in the DMEM medium. The cells were resuspended in the basal experimental medium containing 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose and 20 mM Tes-buffer (pH 7.4), divided into different tubes and spun down in an Eppendorff centrifuge at 10000 × g for 30 s. The medium was carefully sucked off and the cells were subsequently stored on ice. Fluorescence measurements were initiated by resuspending one pellet into the experimental medium containing 1 mM $CaCl_2$ at 37°C and the fluorescence was recorded at 340 nm (ex.) and 505 nm (em.) in a Hitachi F-4000 spectrofluorometer with constant stirring. The dye response was calibrated by sequential addition of 0.1 mg/ml digitonin and 100 μM $MnSO_4$ at the end of the experiment to obtain maximal and minimal fluorescence values respectively [11]. Digitonin was used instead of Triton X-100 for permeabilization purposes since this substance only releases cytoplasmic components when used at low concentrations [12].

Triton X-100 gave about 20% higher maximal fluorescence values which might suggest that organelles had entrapped the dye to a small extent [13]. Calibration of the fura-2 response as a function of the free Ca^{2+} was performed using a H-EDTA (20 mM)/Ca^{2+} (0.5–5 mM) buffer system in supernatants derived from digitonin treated cells. The pH was kept constant by adding NaOH to neutralize protons released by Ca^{2+} binding to H-EDTA. A value of 220 nM was obtained for the apparent Ca^{2+}-fura-2 dissociation constant when previously measured [14] dissociation constants for the H-EDTA-Ca^{2+} were used for the calculation of free Ca^{2+} concentrations (not shown). This value is similar to that obtained by Grynkiewicz et al. [15] in different experimental conditions.

Entrapment of the Ca^{2+} chelator BAPTA into the cells was performed by incubation of cells for 20 min in the basal medium at 37°C containing 1 mM $CaCl_2$, 10 mM glucose and 100 μM BAPTA/AM. Thereafter the cells were washed once in the basal medium, spun down and stored on ice as above.

Measurements of membrane potential using $DiBaC_4$-(3) [16] were performed by washing dissociated cells once in the Na^+-based medium containing 10 mM glucose followed by resuspension into the same medium and centrifugation with an Eppendorff centrifuge as described above. Pellets were subsequently resuspended into the Na^+-based medium at 37°C in the presence of 1 mM $CaCl_2$ and 0.5 μM $DiBaC_4$-(3) and the fluorescence was recorded at 495 nm (ex.) and 517 nm (em.) and additions of effectors were added when a fairly stable fluorescence was obtained within approximately 10–20 min.

3. RESULTS

The effect of carbachol on the membrane potential and the intracellular free Ca^{2+} concentration as measured with $DiBaC_4$-(3) and fura-2, respectively, is shown in fig.1. Carbachol caused an increase in the fluorescence of the bisoxonol, suggesting a depolarization of the membrane. Selective muscarinic receptor agonists, oxotremorine and muscarine, also caused increeases in the bisoxonol fluorescence (not shown). A subsequent addition of gramicidin, which forms nonselective cation channels in the membrane [17] and causes total depolarization [18,19], after carbachol, induced a further increase in the fluorescence of the probe (fig.1). If the Na^+ concentration in the experimental medium was reduced and replaced by choline the effect of carbachol decreased (fig.1b and c). In the absence of Na^+ carbachol had no effect on the fluorescence of the bisoxonol (fig.1d). Readdition of Na^+ stepwise to 60 mM caused only a small increase in fluorescence. Gramicidin, however, increased the fluorescence significantly after 60 mM Na^+ had been readded to the medium.

The resting free Ca^{2+} concentration in SH-SY5Y neuroblastoma cells was 120 ± 24 nM (± SD, n = 10). Carbachol caused a transient increase in the fura-2 fluorescence indicating a rise in cytosolic free Ca^{2+} (fig.1e). The Ca^{2+} transient started immediately to decline and reached the resting value within about 1 min. In the Na^+-free medium the carbachol-induced increase in cytosolic Ca^{2+} was reduced by 20–30% (fig.1f).

In order to examine whether there was a link between the increase in cytosolic Ca^{2+} and depolarization, additions were made of the Ca^{2+} ionophore, ionomycin at a concentration (80 nM) which caused a similar rise in cytosolic Ca^{2+} (fig.2a and b) as carbachol. Ionomycin caused a depolarization to about the same extent as that induced by carbachol (fig.2c and d). Only a small further change in the bisoxonol fluorescence could be obtained when carbachol was added after ionomycin and vice versa. Depletion of intracellular Ca^{2+} pools by the combined addition of

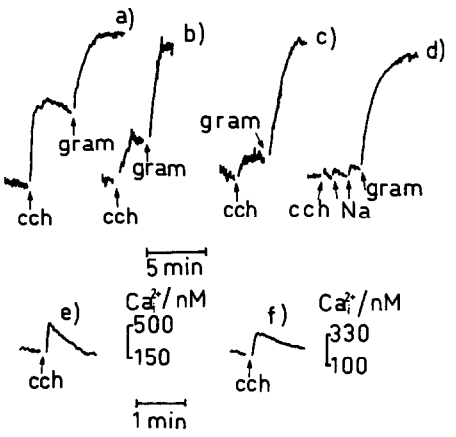

Fig.1. Na^+-dependence of carbachol-induced increase in DiBa-C_4-(3) and fura-2 fluorescence of SH-SY5Y cells. See section 2 for experimental details. In traces a–d the DiBa-C_4-(3) fluorescence changes were recorded in response to 100 μM carbachol (cch) and 0.5 μM gramicidin (gram) in the Na^+-based medium (a), in 70 mM NaCl (b), 40 mM NaCl (c), or in a medium where NaCl had been totally replaced by choline chloride (d) and additions were made of 100 μM carbachol, 30 mM NaCl (Na) twice followed by 0.5 μM gramicidin. In e and f the fura-2 fluorescence was recorded and additions were made of 100 μM carbachol in the Na^+-based (e) or choline-based (f) medium respectively. An upward deflection denotes an increase in cytosolic Ca^{2+} or depolarization.

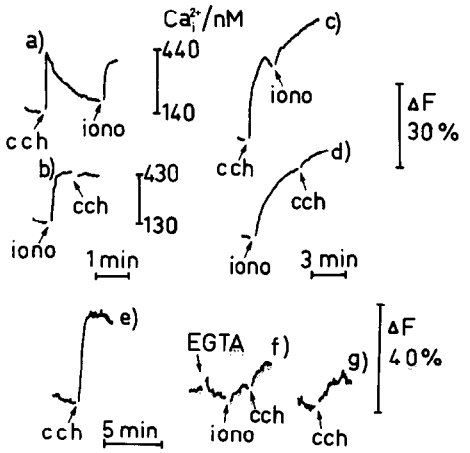

Fig.2. Effect of ionomycin, Ca^{2+}-depletion and BAPTA entrapment on carbachol-induced increase in DiBa-C_4-(3) fluorescence in SH-SY5Y cells. (a,b) Fura-2 recordings in response to 100 μM carbachol (cch) and 80 nM ionomycin (iono) respectively. (c,d) Similar additions in the presence of DiBa-C_4-(3). (e) Addition of 250 μM carbachol; (f) addition of 2 mM EGTA, 1 μM ionomycin and 250 μM carbachol; (g) addition of 250 μM carbachol to BAPTA loaded cells. The scales on the right indicate the percentage of increase in fluorescence as compared to the total fluorescence change after the addition of gramicidin (100%) as in fig.1.

EGTA and ionomycin [20] considerably reduced the increase in fluorescence caused by carbachol (fig.2f). Only a small increase in the bisoxonol fluorescence was observed upon addition of carbachol (fig.2g) to cells that had been loaded with the Ca^{2+} chelator BAPTA [21]. The activator of protein kinase C, TPA, had no effect on the bisoxonol fluorescence (not shown).

The dose-response relationship for carbachol-induced bisoxonol fluorescence and increased fura-2 fluorescence is shown in fig.3. The ED_{50} value for the fura-2 signal was about 4 μM while that of the bisoxonol response was around 20 [4]. An 1,3,4 IP_3-induced effect is thus not a likely mechanism to explain the main part of carbachol-induced depolarization observed here since only a small effect of carbachol was obtained after ionomycin.

Depolarization in response to TPA-induced activation of protein kinase C due to reduction in K^+ conductance has been described in the NG108-15 neuroblastoma/glioma hybrid cell line [2]. Since TPA had no effect on the bisoxonol fluorescence in the presence of SH-SY5Y cells a protein kinase C-linked mechanism could not explain the obser-

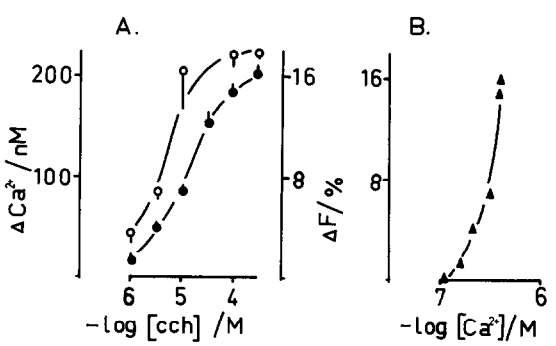

Fig.3. Dose-response relationship of the effect of carbachol on the fura-2 and DiBa-C_4-(3) fluorescence. Conditions were as in figs 1 and 2. (A) Different concentrations of carbachol were added and the fura-2 (○) or DiBa-C_4-(3) fluorescence was recorded. The bisoxonol response (●) is calculated as percent increase in fluorescence as compared to a maximal change caused by gramicidin. (B) The bisoxonol fluorescence is plotted as a function of the peak of the intracellular free Ca^{2+} concentration.

vations made here with carbachol. TPA, however, causes depolarization of primary astrocytes through a decrease in the K^+ permeability [24] which might suggest that the TPA-induced depolarization could be due to the glioma part of the hybrid cell.

The Na^+ dependent depolarization by carbachol observed here could be due to an increase in Na^+ flow into the cells through Ca^{2+}-activated nonselective cation channels like those described by Yellen [5]. The lack of an effect of Na^+ readdition after carbachol on the membrane potential is probably due to the high Na^+ dependency of the carbachol-induced potential change, the transient change in Ca^{2+} and the slow response-time of the bisoxonol [16]. The possibility that Na^+ ions are required for the potential changes through some other mechanisms cannot, of course, be excluded.

The nonselective cation channels are optimally activated by μmolar Ca^{2+} concentrations [5,25,26] which might explain the apparent lack of saturation of the response obtained here since the peak rise in Ca^{2+} in response to carbachol was around 500 nM and thus the putative Ca^{2+} binding site would be far from saturation. The Ca^{2+} activated channel could thus in concert with a reduction in the K^+ permeability be the mechanism of muscarinic receptor-linked changes in membrane potentials. Ca^{2+} activated nonselective channels have also been observed in heart cells [25,26] and other cell types [26]. In heart cells the nonselective channel has been suggested to play a role in pacemaking activity.

In conclusion the results suggest that an increase in intracellular Ca^{2+} in response to the addition of carbachol depolarizes SH-SY5Y cells through a Na^+-dependent mechanism.

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