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Demonstration of a Muscarinic Receptor-Mediated Cyclic GMP-Dependent Hyperpolarization of the Membrane Potential of Mouse Neuroblastoma Cells Using [^{3}H]Tetraphenylphosphonium

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SUMMARY

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The lipophilic cation [^{3}H]tetraphenylphosphonium ([^{3}H]TPP) was used to measure the transmembrane potential (V_m) of cultured mouse neuroblastoma cells (clone N1E-115) in suspension. These cells accumulated approximately twice as much [^{3}H]TPP in low-K^+ phosphate-buffered saline (PBS) as they did in high-K^+ PBS. Accumulation in the presence of either low or high potassium was both time- and temperature-dependent. At equilibrium, [^{3}H]TPP accumulation in low-K^+ and high-K^+ PBS increased with increasing cell number, and net accumulation increased linearly with the external [^{3}H]TPP concentration between 0.1 and 50 μM. Under equilibrium conditions at 37°, the addition of 1 mM carbachol significantly increased net [^{3}H]TPP accumulation from 519 ± 30 pmoles/10^6 cells to 1160 ± 33 pmoles/10^6 cells within 1 min. This increase was equivalent to a hyperpolarization of the cells' V_m from approximately −66 ± 5 mV to −79 ± 5 mV. Direct measurements with microelectrodes under these same conditions showed that there was an immediate and significant hyperpolarization of the cells' V_m from −62.3 ± 0.5 mV to −72.0 ± 1.3 mV. Atropine (1 μM), but not *d*-tubocurarine (10 μM) or pyrilamine (10 μM) prevented the increase in [^{3}H]TPP accumulation. This agonist-mediated hyperpolarization was abolished either by adding ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid to the cells or by using a Ca^{2+}-free buffer. Under similar conditions, cyclic GMP increased net [^{3}H]TPP accumulation to 1050 ± 31 pmoles/10^6 cells within 10 min (i.e., an increase equivalent to a hyperpolarization of the cells' V_m from −66 ± 5 mV to −76 ± 6 mV). Direct electrophysiological measurements under these same conditions showed that there was a significant hyperpolarization of the cells' V_m from −62.3 ± 0.5 mV to −71.2 ± 1.5 mV after a period of 3.8 ± 0.6 min. These data suggest that muscarinic receptor responses in these cells may be mediated by a hyperpolarization of the cells' V_m subsequent to an increase in intracellular cyclic GMP.

INTRODUCTION

For those drugs that interact with receptors, the pharmacodynamic aspects of their action have been divided into three main phases: phase 1, the interaction of the drug with its receptor to produce a signal; phase 2, the transduction, modulation, and amplification of that signal; and phase 3, the generation of a biological effect. Phases 1 and 3 have been studied extensively and much information is known about them (1). Phase 2, however, is perhaps the most complex and least understood of the three. The purposes of the present study were to determine whether a specific drug-receptor interaction could produce a measurable electrophysiological response and whether that response could be measured biochemically. We chose cultured mouse neuroblastoma cells (clone N1E-115) for these experiments because they have been well studied biochemically, they possess a number of neurotransmitter receptors similar to those found in the mammalian central nervous system, and they provide a readily available, homogeneous cell population that is easy to grow (2).

It is usually necessary to impale cells with microelectrodes to measure directly their transmembrane potential

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(V_m). Lichtshtein *et al.* (3) used [^{3}H]TPP[4], a lipophilic cation that is well characterized chemically (4–7) and has been used to measure the V_m of mitochondria (8), Ehrlich's ascites cells (9), and neuroblastoma-glioma hybrid cells (clone NG-108-15). Similar lipophilic ions have also been used to study various prokaryotic and eukaryotic cells (10–13). In the present study, we characterized the accumulation of [^{3}H]TPP by N1E-115 mouse neuroblastoma cells and used this method to demonstrate Ca^{2+}- and cyclic GMP-dependent, muscarinic receptor-mediated hyperpolarization of the cells' V_m that was verified, under identical experimental conditions, by direct measurements with microelectrodes.

MATERIALS AND METHODS

An initial batch of [^{3}H]TPP (2.51 Ci/mmole) was supplied graciously by Drs. H. R. Kaback and A. J. Blume (Roche Institute of Molecular Biology). Additional [^{3}H]TPP (4.79 Ci/mmole) was synthetized by New England Nuclear Corporation, Boston, Mass., by the following method: Analytical grade TPP^+Br^- (71.9 mg) (Ventron-Alfa Division, Danvers, Mass.) was dissolved in dimethyl formamide (0.3 ml) and triethylamine (0.1 ml); 5% RH/Al_2O_3 catalyst (50 mg) and 25 Ci of [^{3}H]H_2O were added to the mixture, which was stirred overnight at 80°. Added hydrogen bromide (0.1 mM) and labile tritium were removed *in vacuo*, using ethanol as a solvent. After filtration from the catalyst, the product was dried *in vacuo* and resuspended in 10 ml of ethanol. Radiochemical purity of the [^{3}H]TPP was determined using reverse isotope dilution analysis.

All other materials were analytical grade and were obtained from commercial sources.

Culture conditions and cell preparation. N1E-115 mouse neuroblastoma cells were grown at 37° in Dulbecco-Vogt's modification of Eagle's medium (Grand Island Biological Company, Grand Island, N. Y.) that was supplemented with 10% (v/v) fetal calf serum (Grand Island Biological Company), without antibiotics, in an atmosphere of 10% CO_2/90% humidified air. Cells (passages 8–19) were grown to confluency in tissue culture flasks (75 cm^2/250 ml, 20-ml medium; Falcon Plastics, Los Angeles, Calif.) and maintained at stationary phase for at least 1 week prior to their use in the experiments.

Cells were dissociated from their flasks by a 10-min incubation (37°) in a modified Puck's D_1 solution, collected by centrifugation (230 × *g* for 1 min in a Damon/IEC CRU-5000 centrifuge), and resuspended in either a low-K^+ PBS containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 25 mM glucose, and 70 mM sucrose (37°, pH 7.4, 340 mOsm) or a high-K^+ PBS containing the same constituents except for the substitution of 100 mM KCl for 5.3 mM KCl and the removal of sucrose.

Cell numbers, the average cell diameter (20 μm), and the average cell volume (7.6 ± 0.3 $\mu l/10^6$ cells) were determined using an electronic cell counter (model Z_F, Coulter Electronics Inc., Hialeah, Fla.).[5]

[4] The abbreviations used are: [^{3}H]TPP, [^{3}H]tetraphenylphosphonium bromide; PBS, phosphate-buffered saline; DNP, 2,4-dinitrophenol.

[5] R. A. Gorkin and E. Richelson, manuscript in preparation.

Measurement of [^{3}H]TPP accumulation. Cellular accumulation of [^{3}H]TPP was initiated by adding cells (0.05 ml), suspended in the appropriate PBS buffer at 37°, to glass test tubes containing a test drug, 25 nM [^{3}H]TPP, and 5 μM TPP in either low-K^+ or high-K^+ PBS (0.5 ml total volume). Accumulation was continued for the specified times at 37°. The incubations were terminated by removing the contents of the test tubes with Pasteur pipettes, layering the contents onto oil (5:1 dibutyl dinonylphthalate; 0.5 ml; upper layer)-sucrose (30% in water; 0.5 ml; lower layer) gradients that were contained in plastic Microfuge tubes (2 ml) and centrifuging these tubes in a Beckman Microfuge B for 1-½ min. The supernatants were removed and discarded and the pellets were resuspended in 1 ml of Triton X-100 (1–2%) by trituration. After 6 hr, this mixture was transferred to counting vials containing 7 ml of Quantafluor (Mallinckrodt Inc., St. Louis, Mo.), and the amount of radioactivity was determined using a Searle Isocap/300 liquid scintillation counter (37% efficiency). Unless specified, all [^{3}H]TPP accumulation experiments were carried out in this manner. The radioactivity trapped in the extracellular space of the pellets was minimal (~1% of the total radioactivity per tube).

Electrophysiological measurements. Transmembrane potentials were recorded intracellularly using glass microelectrodes drawn from WPI capillary tubing (1.5 mm outer diameter; 1.0 mm inner diameter) that contained filament. The electrodes were filled with 3 M KCl, and only those with a tip potential of less than −5 mV and a resistance of 28–35 MΩ were used. The microelectrodes were connected to a high-input impedance preamplifier (Bioelectric Instruments, Chicago, Ill, Model PAD 2A).

Cells suspended in the appropriate PBS buffer were poured into a Lucite chamber (2-ml volume) and mounted on the stage of a Wild microscope (× 10 objective). The temperature of the buffer (37°) was maintained using a Peltier temperature controller. To facilitate the changing of buffering solutions, a suction device and a plastic syringe were placed on opposite sides of the chamber, and the vacuum and injection rates were adjusted so that all of the fluid in the chamber could be replaced within 1 sec while a given cell was impaled.

Analysis of biochemical data. Direct microelectrode recordings from these neuroblastoma cells (14) and others (3) showed that high-K^+ media abolish the V_m, indicating that [^{3}H]TPP accumulation under these circumstances is not related to the V_m (i.e., these cells are nonpolarized in high-K^+ media because their transmembrane potential is essentially equal to zero). By subtracting the amount of [^{3}H]TPP accumulation in a high-K^+ medium from that in a low-K^+ medium, therefore, one can obtain that portion of the total accumulation that is related to the V_m (i.e., $[^3\text{H-TPP}]_{in}^{corr}$). This component can be divided by the average cell volume, which increases by only approximately 20% in high-K^+ PBS[5], to obtain the intracellular TPP concentration. When the intracellular concentration is divided by the external TPP concentration, the resultant concentration ratio (R_{TPP}) can be used in the Nernst equation ($V_m = -61 \log R_{TPP}$) to calculate the cells' V_m. All biochemical data from the present study were analyzed in this manner.

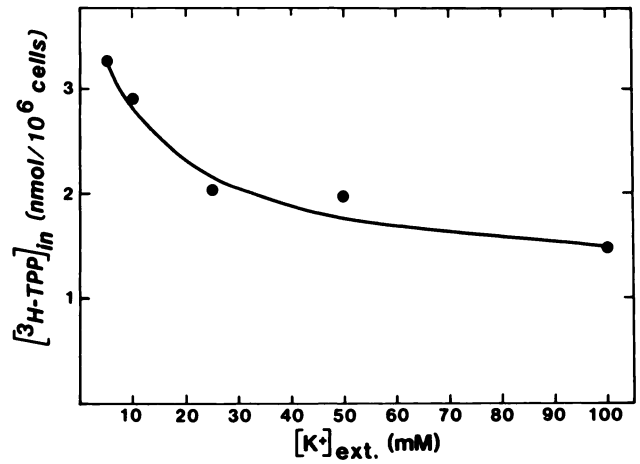

FIG. 1. *Dependence of [^{3}H]TPP accumulation by N1E-115 mouse neuroblastoma cells on the external K^+ concentration*

RESULTS

[^{3}H]TPP accumulation. N1E-115 mouse neuroblastoma cells accumulated approximately twice as much [^{3}H]TPP in low-K^+ PBS (5.3 mM) as they did in high-K^+ PBS (100 mM) (Figs. 1 and 2). This accumulation was time- and temperature-dependent and equilibrated within 30 min in both low- and high-K^+ PBS at 37° (Fig. 2). The equilibrium $t_{1/2}$ for [^{3}H]TPP accumulation in low-K^+ PBS was 5.0 min, that in high-K^+ PBS was 1.5 min, and the $t_{1/2}$ for net [^{3}H]TPP accumulation was 4.8 min.

Net [^{3}H]TPP accumulation, measured at 0° after a 37° preincubation for 10 min, required approximately 10 min

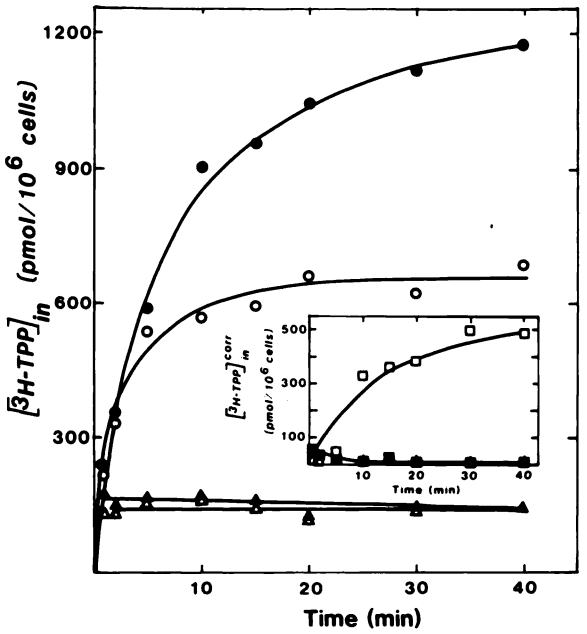

FIG. 2. *Time and temperature dependence of [^{3}H]TPP accumulation by N1E-115 mouse neuroblastoma cells in suspension*

●, Accumulation at 37° in low-K^+ PBS; ○, accumulation at 37° in high-K^+ PBS. ▲, △, Accumulations at 0° in low-K^+ PBS and high-K^+ PBS, respectively. Each symbol includes the mean value ± standard error of the mean. In the *inset*, □, net [^{3}H]TPP accumulation at 37°; ■, that at 0°.

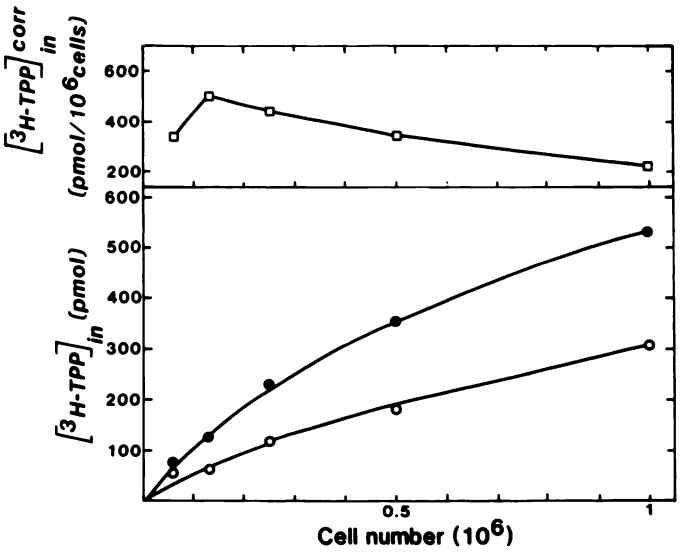

FIG. 3. *Dependence of [^{3}H]TPP accumulation by N1E-115 neuroblastoma cells on cell number*

●, Accumulation at 37° in low-K^+ PBS; ○, that in high-K^+ PBS. □, Net [^{3}H]TPP accumulation at 37°.

to decrease from 50 pmoles/10^6 cells to 2 pmoles/10^6 cells (Fig. 2, *inset*). Immersing the cells in an ice bath, therefore, effectively stopped [^{3}H]TPP accumulation. Rapid cooling was used subsequently when it became necessary to measure accumulation after very short periods of time.

Equilibrium [^{3}H]TPP accumulation in low-K^+ PBS and high-K^+ PBS increased with increasing cell number. At the cell concentrations tested, net accumulation was highest (500 ± 28 pmoles/10^6 cells) at 0.25 × 10^6 cells/ml and lowest (225 ± 31 pmoles/10^6 cells) at 2 × 10^6 cells/ml (Fig. 3). Net [^{3}H]TPP accumulation increased linearly with the external TPP concentration between 0.1 and 50 μM (Fig. 4). Ouabain (1 mM) and DNP (1 mM) inhibited net [^{3}H]TPP accumulation by approximately 30% and 50%, respectively (data not shown). By substituting data derived from [^{3}H]TPP accumulation studies into the

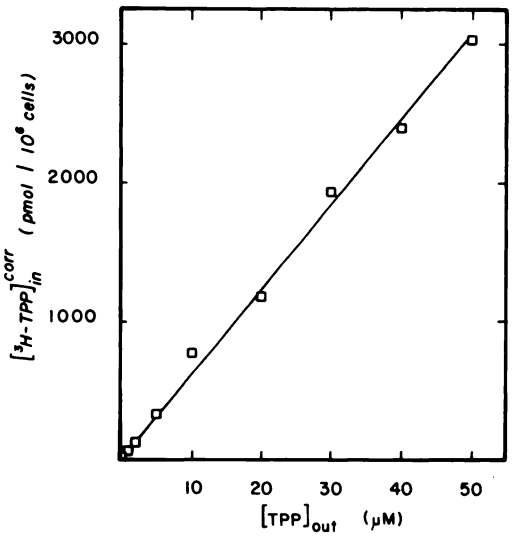

FIG. 4. *Dependence of net [^{3}H]TPP accumulation by N1E-115 neuroblastoma cells on the external TPP concentration*

Nernst equation, a resting V_m of −67 ± 5 mV was calculated for these cells.

After [^{3}H]TPP accumulation had equilibrated at 37° (i.e., 519 ± 30 pmoles/10^6 cells at $t \geq 30$ min), the addition of carbachol (1 mM, the experimental concentration that was found to produce the maximal cellular response; Fig. 5) produced an increase in the net [^{3}H]TPP accumulation (1160 ± 33 pmoles/10^6 cells) within 1 min that returned to control levels within 7 min (Fig. 6). This increase represented a statistically significant ($p < 0.05$) hyperpolarization of the cells' membranes from −66 ± 5 mV to −79 ± 5 mV. When carbachol was added to the cell suspension at the beginning of the incubation period ($t = 0$), there was no increase in net [^{3}H]TPP accumulation above control levels (Fig. 6). The addition of atropine (1 μM), but not *d*-tubocurarine (10 μM) or pyrilamine (10 μM), to the cell suspension prevented this carbachol-induced increase in net [^{3}H]TPP accumulation (data not shown). This agonist-mediated hyperpolarization could be abolished either by adding 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid to the cell suspension or by using a Ca^{2+}-free buffer (data not shown).

Under equilibrium conditions at 37°, the addition of 1 mM cyclic GMP to the cell suspension increased net [^{3}H]TPP accumulation to 1050 ± 31 pmoles/10^6 cells within the specified 10-min period (Fig. 6). This increase represented a statistically significant ($p < 0.05$) hyperpolarization of the cells' V_m from −66 ± 5 mV to −76 ± 6 mV.

Electrophysiological measurements. For an electrophysiological measurement to be acceptable in our study, the V_m must have been stable for at least 10 sec following cell penetration. The recorded V_m values were independent of the microelectrode's resistance (28–35 MΩ), indicating that these measurements did not result from salt leakage from the microelectrode.

Cells studied electrophysiologically under identical experimental conditions yielded V_m values of −63.0 ± 0.6 mV ($n = 16$) and −3.1 ± 0.8 mV ($n = 15$) in low-K^+ and high-K^+ PBS, respectively.

In confirmation of previous results, we found that cells in suspension (V_m = −63.0 ± 0.6 mV) were more internally electronegative than plated cells (V_m = −36 to −47 mV) (3, 14–17).

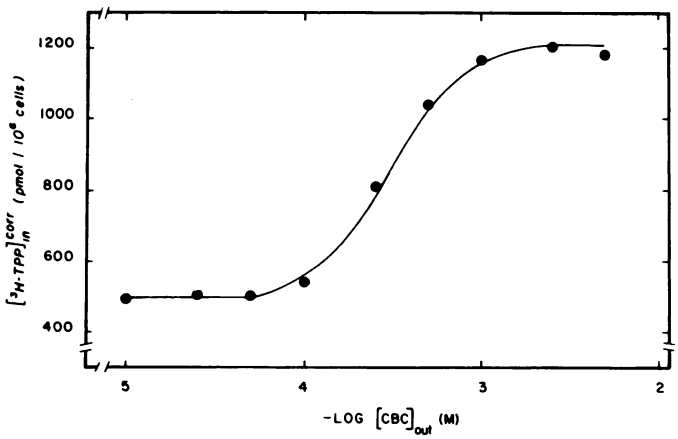

FIG. 5. *Effect of increasing carbachol (CBC) concentration on net [^{3}H]TPP accumulation by N1E-115 neuroblastoma cells*

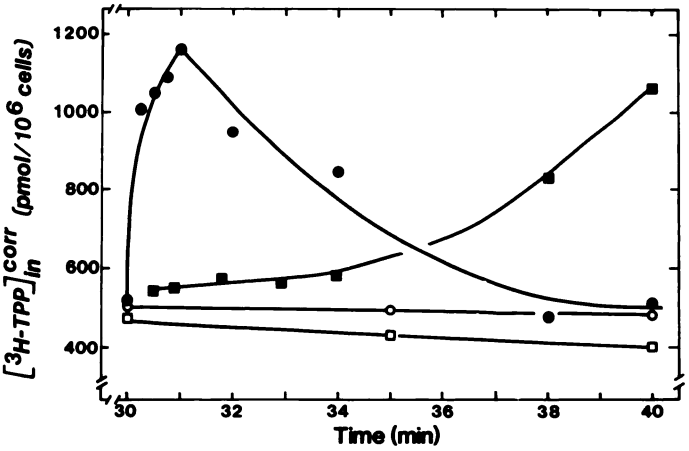

FIG. 6. *Effect of 1 mM carbachol and 1 mM cyclic GMP on net [^{3}H]TPP accumulation by N1E-115 neuroblastoma cells with time*

●, Net accumulation in the presence of carbachol at $t \geq 30$ min. □, Net accumulation in the presence of carbachol (1 mM) from $t = 0$; ■, net accumulation in the presence of cyclic GMP at $t \geq 30$ min. ○, Control net accumulation values.

When cells that were impaled with microelectrodes for the measurement of V_m were exposed to PBS containing carbachol (1 mM), there was an immediate and statistically significant ($p < 0.01$) hyperpolarization of their membranes from −62.3 ± 0.5 mV to −72.0 ± 1.3 mV. When cells from sister flasks were exposed to PBS containing cyclic GMP (1 mM), there was a statistically significant ($p < 0.01$) hyperpolarization of their membranes from −62.3 ± 0.5 mV to −71.2 ± 1.5 mV after a period of 3.8 ± 0.6 min (Table 1).

DISCUSSION

The primary purpose of this study was to determine whether a drug-receptor interaction could produce a biochemically measurable electrophysiological response in N1E-115 mouse neuroblastoma cells. Even though Lichtshtein *et al.* (3) had demonstrated previously the usefulness of [^{3}H]TPP accumulation in measuring the V_m of NG108-15 cells, it was first necessary for us to characterize the accumulation of [^{3}H]TPP by N1E-115 cells.

Our electrophysiological data showed that high-K^+ media abolished the V_m of the cells tested and that [^{3}H]TPP accumulation under these circumstances was not due to the V_m. The 2-fold increase in [^{3}H]TPP accumulation in low-K^+ PBS over that in high-K^+ PBS,

TABLE 1

Effect of carbachol and cyclic GMP on the V_m of N1E-115 cells

Results are expressed as means ± standard error of the mean.

Treatment	*n*	V_m
		mV
Control	15	−62.3 ± 0.5
Carbachol (1 mM)	10	−72.0 ± 1.3[a]
Cyclic GMP (1 mM)	10	−71.2 ± 1.5[b]

[a] Significantly different from control ($p < 0.01$). Latency period from application of carbachol to response = 0 min.

[b] Significantly different from control ($p < 0.01$). Latency period from application of cyclic GMP to response = 3.8 ± 0.6 min.

however, did represent accumulation that was due to the V_m (Fig. 2). The [^{3}H]TPP accumulation that was measured in high-K^+ PBS probably represents accumulation into internally electronegative, intracellular organelles (e.g., mitochondria) that are not responsive to the extracellular K^+ concentration (8).

[^{3}H]TPP accumulation is probably not due to simple diffusion because it was temperature-dependent, and ouabain and DNP inhibited net [^{3}H]TPP accumulation by approximately 30% and 50%, respectively. These data indicate either that [^{3}H]TPP accumulation depends somewhat upon energy derived from the hydrolysis of ATP by (Na^+-K^+)-ATPase or that ouabain itself alters the V_m. However, ouabain (1 mM) depolarizes attached N1E-115 cells by only 5.5 mV (18). The inhibition of [^{3}H]TPP accumulation by DNP is another indication that the accumulation may be energy-dependent. Because DNP is a lipophilic acid that promotes H^+ entry into mitochondria (19), however, it may also have an additional direct effect on the V_m.

As the cell concentration increased, the [^{3}H]TPP accumulation in high-K^+ PBS became proportionately larger, such that net accumulation was highest at 0.25×10^6 cells/ml and lowest at 2×10^6 cells/ml. This situation is analogous to the disproportionate increase in the nonspecific binding, and the consequent decrease in specific binding due to the depletion of free ligand, of some radioligands at very high tissue concentrations (20). The decrease in net accumulation at the lowest cell concentration tested was an artifact of the centrifugation of such a small number of cells. It has been shown with direct microelectrode recordings that both cell number and temperature have minimal effects on the V_m of N1E-115 cells (18).

At 37°, using the optimal cell concentration of 0.25×10^6 cells/ml, our biochemical ($V_m = -67 \pm 5$ mV) and electrophysiological ($V_m = -63.0 \pm 0.6$ mV) data agree remarkably well and indicate that [^{3}H]TPP accumulation can be used to measure accurately the V_m of N1E-115 cells. The fact that these cells are essentially nonpolarized in high-K^+ PBS ($V_m = -3.1 \pm 0.8$ mV) indicates that they act as K^+ electrodes. Tuttle and Richelson (14) demonstrated that when the V_m values of N1E-115 cells were measured in a series of solutions with varying $[K^+]_{ext}$ and the data were plotted with $e^{V_m F/RT}$ on the ordinate and $[K^+]_{ext}$ on the abscissa, these two variables had a linear relationship over $[K^+]_{ext}$ of 20 to 100 mM. These data, combined with those of Lichtshtein *et al.* (3), justify the assumptions that (1) the difference in [^{3}H]-TPP accumulation in high-K^+ PBS from [^{3}H]TPP accumulation in low-K^+ PBS represents the portion of [^{3}H]1 72TPP accumulation that is related to the V_m, and (2) R_{TPP} TPP accumulation that is related to the V_m, and (2) R_{TPP} can be used in the Nernst equation to calculate the cells' V_m (see Results).

Carbachol increased [^{3}H]TPP accumulation (indicating a hyperpolarization of the cell membranes from -65 ± 5 mV to -79 ± 6 mV) when it was added to the cell suspensions after the [^{3}H]TPP accumulation had equilibrated. When carbachol was incubated with the cells from t_0, there was no increase in [^{3}H]TPP accumulation at 30 min because the prolonged exposure to the drug probably desensitized the receptor (21, 22), as suggested by the decline in [^{3}H]TPP accumulation after the agonist-induced increase had occurred. The fact that atropine (1 μM), but not *d*-tubocurarine (10 μM) or pyrilamine (10 μM), prevented this increase in accumulation indicates that the phenomenon is muscarinic receptor-mediated.

Electrophysiological measurements made under identical experimental conditions revealed similar hyperpolarization of the membranes from -62.3 ± 0.5 mV to -72.0 ± 1.3 mV upon exposure to carbachol. The fact that carbachol hyperpolarized, rather than depolarized, these cells is not surprising since muscarinic inhibition has been demonstrated micro-iontophoretically in many tissues of the mammalian central nervous system (23). Replacement of the medium bathing the cells never produced a change in the V_m greater than ± 2 mV. Therefore, the recorded hyperpolarization was not due to the replacement of the medium per se. After the addition of carbachol, the hyperpolarization was instantaneous and remained constant during the 30-sec recording period. The increase in [^{3}H]TPP accumulation, under the same conditions, was not instantaneous because (1) for there to be an increase in accumulation, the inside of the cell had to become more electronegative and the carbachol-induced hyperpolarization of the membrane would have had to precede the increase; and (2) the experimental manipulations of the biochemical assay required more time than that required for changing the cells' bathing medium. These two factors may limit to some extent the usefulness of [^{3}H]TPP in measuring very rapid changes in V_m.

Muscarinic receptor-mediated formation of cyclic GMP is believed to play a role in the postsynaptic transfer of information between cells of neural origin (including neuroblastoma cells) (24, 25). Richelson and Divinetz-Romero (26) showed that carbachol stimulates cyclic GMP formation in N1E-115 neuroblastoma cells, and Richelson *et al.* (27) showed that this stimulation is dependent upon the external Ca^{2+} concentration. We found that the addition of cyclic GMP (1 mM) to a suspension of N1E-115 cells increased [^{3}H]TPP accumulation, representing a hyperpolarization of the cells' V_m similar to that caused by carbachol but with a slower time-course. This lag in the response time to cyclic GMP was most likely due to the time necessary for cyclic GMP to diffuse into the cells (i.e., since cyclic AMP enters cells, it is likely that cyclic GMP does so as well) (28). Direct microelectrode recordings, made under identical experimental conditions after the application of cyclic GMP, showed a hyperpolarization of the cells' V_m from -62.3 ± 0.5 mV to -71.2 ± 1.5 mV after a latent period of 3.8 ± 0.6 min. The observation that the hyperpolarization caused by cyclic GMP showed a latent period of approximately 10 min in the biochemical assay and only approximately 4 min in the electrophysiological study may be explained by the fact that, in this case, both cyclic GMP and [^{3}H]TPP must diffuse across the cells' membranes before a change in V_m can be measured. Once again, these results point to the limitations of measuring the time-course of changes in V_m using [^{3}H]TPP accumulation. Given these data, however, it appears that

activation of muscarinic receptors in these cells results in an increase in cyclic GMP which in turn causes a hyperpolarization of the cells' V_m. Further support for this conclusion stems from the observation that this muscarinic receptor-mediated hyperpolarization was abolished under conditions where no cyclic GMP was formed (i.e., either in the presence of 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid or in a Ca^{2+}-free buffer).

In conclusion, the data presented here in show that [^{3}H]TPP accumulation by N1E-115 mouse neuroblastoma cells can be used to measure a Ca^{2+}- and cyclic GMP-dependent muscarinic receptor-mediated hyperpolarization of the cells' V_m. Future studies of this type should prove to be valuable in obtaining more detailed information about the transduction of neurotransmitter signals at postsynaptic receptors.

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