Agonist-induced Changes in the
Modulation of K⁺ Permeability and
Beating Rate by Muscarinic Agonists
in Cultured Heart Cells

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ABSTRACT  The correlation between number of muscarinic cholinergic receptor sites as measured by binding of the muscarinic antagonist [³H]methylscopolamine ([³H]MS) and the ability of muscarinic agonists to mediate a physiologic response was determined in intact heart cells cultured from chick embryos 10 d in ovo. The increase in K⁺ permeability and the decrease in beating rate mediated by the muscarinic agonist carbachol were the responses studied. Exposure to 10⁻³ M carbachol caused a 15% decrease in beating rate and a 33% increase in the rate of ⁴²K⁺ efflux from cells labeled to equilibrium. An assay for binding of [³H]MS to intact cells was developed. [³H] MS bound specifically to intact heart cells (185 fmol/mg protein) with a Kd of 0.48 nM. Exposure of cells for various times to 10⁻³ M carbachol followed by binding of [³H]MS to intact cells demonstrated that a gradual loss of 70% of [³H]MS binding sites took place over the next 6 h with a T₁/₂ of 30 min. A decrease in the ability of carbachol to stimulate K⁺ efflux and to decrease beating rate was observed after pre-exposure of cells to muscarinic agonists. A close correlation was found between the loss of the subclass of muscarinic receptors subject to agonist control and the loss of physiologic responsiveness after agonist exposure. The data suggest the absence of significant numbers of “spare” receptors within this group.

INTRODUCTION

The ability of hormone-sensitive cells to respond to changes in hormonal concentration by regulating the number and/or affinity of their cell surface receptors has been demonstrated in cells regulated by neurotransmitters, peptide and protein hormones, and factors such as lectins and immunoglobulins (Raff, 1976). The effect of such changes in receptor number and affinity on the physiologic response of the cell to hormone or neurotransmitter
stimulation has been a subject of considerable interest (Catt et al., 1979). Even in the presence of “spare” receptors not obviously coupled to a physiologic response, reduction in membrane receptor number below a critical level would be expected to decrease the sensitivity of the cell to the effector. In luteal cells from rat ovaries in which hormone action mediates changes in adenylate cyclase activity, a close correlation has been demonstrated between luteinizing hormone (LH)-induced decrease in LH receptors and a decrease in the ability of LH to stimulate the synthesis of cyclic AMP and progesterone (Conti et al., 1977). In frog erythrocytes, an agonist-induced decrease in β-adrenergic agonist binding sites has been correlated with a decrease in catecholamine-stimulated adenylate cyclase activity (Wessels et al., 1978).

Recently, experiments using the potent muscarinic antagonist [3H]-quinuclidinyl benzilate ([3H]QNB) for the measurement of muscarinic binding sites have demonstrated that prior exposure of neuroblastoma cells (Siman and Klein, 1979) or cultures of embryonic chick heart cells (Galper and Smith, 1980) to muscarinic cholinergic agonists decreased the number of muscarinic binding sites to as low as 30% of control levels. The response of embryonic chick heart cell cultures to muscarinic agonists revealed three subclasses of receptor sites: [3H]QNB binding sites that are lost during the first minute of agonist exposure, constituting 26% of the total, accompanied by a decrease in apparent affinity of all remaining receptors for agonist; [3H]QNB binding sites that are lost over 2.5 h of agonist exposure, constituting 43% of the total; and receptors that continued to bind [3H]QNB after 3 h of agonist exposure, making up 30% of the total (Galper and Smith, 1980). Whether any of these changes in receptor number are coupled to a decrease in physiologic response to muscarinic agonist has not been determined.

Muscarinic cholinergic stimulation of the heart causes a decrease in the rate and force of contraction. In atrial and nodal tissue, vagal stimulation or application of exogenous acetylcholine has been associated with hyperpolarization of the resting membrane potential (Glitsch and Pott, 1978) and a shortening of the action potential duration. Both of these effects have been considered to be the consequence of the development of an outward K+ current (Antoni and Rotmann, 1968) whose magnitude is determined by the frequency of vagal stimulation or the concentration of exogenously added acetylcholine (Garnier et al., 1978; Osterrieder et al., 1980) and which accounts for the decrease in the rate and force of contraction. Harris and Hutter (1956) were the earliest to demonstrate a vagally mediated increase in efflux of 42K+ from tortoise and frog sinus venosus. However, more recently, Giles and Noble (1976) and others have presented evidence that in atrial tissue the negative inotropic effect could also be mediated by a direct inhibitory effect of acetylcholine on the slow inward current. Hino and Ochi (1980) reported that in ventricular myocardium acetylcholine exerted its effect primarily by decreasing the slow inward current with no detectable increase in the outward K+ current.

In the present investigation we studied the relationship between changes in the properties and number of muscarinic receptors and the beating rate and
Control of Muscarinic Response in Cultured Heart Cells

K⁺ permeability responses to muscarinic agonists in cultured heart cells. Specifically, we determined the ability of muscarinic agonists to elicit these responses before and after receptor number was altered by prior exposure to muscarinic agonists. We also examined the relationship between changes in a particular subclass of receptors (Galper and Smith, 1980) and altered K⁺ permeability and beating rate responses.

METHODS

Materials

Chemicals were obtained from the following sources: carbamylcholine chloride from Sigma Chemical Co., St. Louis, MO; oxotremorine from Aldrich Chemical Co., Milwaukee, WI; medium M-199 from Microbiological Associates, Walkersville, MD; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) from Calbiochem-Behring Corp., San Diego, CA; [³H] methyl-scopolamine ([³H]MS) (53 Ci/mM) and ⁴²K⁺ from New England Nuclear, Boston, MA. Embryonated Leghorn chicken eggs (flock MR 58) were obtained from Spafas, Inc. Norwich, CT. Fetal calf serum was from North American Biological, Miami, FL.

Media

Cells were grown in a modification of medium M-199 consisting of either 40% (vol/vol) M-199, 54% (vol/vol) Earle’s salt solution, and 6% heat-inactivated (30 min, 57°C) fetal calf serum, or 20% (vol/vol) M-199, 74% (vol/vol) Earle’s salt solution, 2% horse serum, and 4% fetal calf serum. Ca⁺² was adjusted to 0.72 mM with CaCl₂.

The medium used for all [³H]QNB binding studies was HEPES-buffered M-199 consisting of 21% (vol/vol) M-199 and 79% (vol/vol) of a buffered salt solution containing 117 mM NaCl, 4.4 mM KCl, 0.8 mM MgSO₄, 25 mM HEPES (adjusted to pH 7.4 with NaOH), 5 mM glucose, 0.001% (wt/vol) phenol red, and 1.8 mM CaCl₂.

Heart Cell Cultures

Heart cell cultures were prepared by a modification of the method of DeHaan (1967) as described previously (Galper and Smith, 1978) except that Ca⁺²-Mg⁺²-free Hanks’ balanced salt solution was used in place of Puck’s Saline G and medium M-199 was prepared as described above.

Hearts were removed, minced, and incubated with 0.025% (wt/vol) trypsin in Ca⁺²-Mg⁺²-free Hanks’ balanced salt solution at 37°C for 8 min. The trypsin solution was removed and diluted into Ca⁺²-Mg⁺²-free balanced salt solution containing 50% heat-inactivated horse serum at room temperature. After successive trypsinizations, suspensions of trypsinized cells were sedimented at 1,000 rpm in a desk-top centrifuge and resuspended in growth medium, and cells used for ion flux studies were plated directly onto 100-mm petri dishes containing 25-mm circular coverglasses (VWR-Vanlab thickness No. 2; VWR Scientific Inc., San Francisco, CA). Cultures were incubated in a humidified 5% CO₂/95% air atmosphere. Confluent monolayers developed by the third day in culture, at which time ion flux, ligand binding, and beating rate studies were performed.

MEASUREMENT OF [³H]MS BINDING TO INTACT CELLS

Cells prepared as described were plated in Costar multiwells (16-mm Diam; Costar, Data Packaging, Cambridge, MA) at 4 × 10⁻⁵ cells per well. On the third culture day, cells were fed with fresh
medium containing 0.01 mCi/ml leucine [14C(U)], and incubation was continued for 24 h. To study the binding of [3H]MS, 0.5 ml of HEPES-buffered M-199 2 nM in [3H]MS was added to each well and incubated at 37°C for 1 h.

Wells were rinsed rapidly three times with wash medium (120 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 50 mM HEPES, and 1.0 mM NaH₂PO₄ adjusted to pH 7.4 with NaOH). 1 ml 0.5 M NaOH was added to each well to solubilize cell proteins, and 0.8-ml aliquots from each well were neutralized with 0.5 ml of Tris pH 7.4 mixed with 10 ml of Instagel (Packard Instrument Co., Downers Grove, IL). 3H and 14C were counted in a liquid scintillation counter. Protein content was determined for one well in four by the method of Lowry et al. (1951), and the mean protein content per 1,000 14C counts was calculated. This factor permitted calculation of protein content in each well. All [3H]MS binding was normalized to specific binding per milligram of cell protein. Total washing time was 10 s. [3H]MS binding was found to be constant during this period. Specific binding was defined as binding inhibited by a saturating concentration (0.1 mM) of oxotremorine or 1.0 mM methyl-scopolamine. Nonspecific binding was determined in quadruplicate for each experimental point. 50% of [3H]MS binding was specific under these conditions. A typical well containing 0.2 mg of protein bound 2,000 cpm of [3H]MS at a counting efficiency of 33%. Under the conditions described, specific binding at 2 nM [3H]MS proceeded without a lag and reached 50% of saturation in 5.0 ± 0.6 min (SEM, n = 3). Binding reached equilibrium by 40 min. The relative potency of cholinergic ligands to inhibit [3H]MS binding was consistent with the relative pharmacologic potency of muscarinic agonists and antagonists (Galeer et al., 1982).

**ION FLUX MEASUREMENTS: 42K⁺ UPTAKE**
Cells grown on coverslips for 3 d were labeled for 24 h with 0.2 μCi/ml leucine [4,5-3H]. As described previously (Biedert et al., 1979), coverslips were placed in an uptake chamber containing fresh medium at 37°C in an atmosphere of 95% air/5% CO₂, transferred to a second chamber with medium containing 42K⁺ (5 μCi/ml, total K⁺ = 4.5), and incubated for the appropriate times. Coverslips were rinsed three times by dipping into Earle's saline solution and cells were then solubilized in 1 ml of 0.5 N NaOH. An aliquot of each sample was taken and neutralized with 0.5 ml of Tris-HCl (pH 7.4), 10 ml of scintillation fluid was added, and 42K⁺ and 3H were determined in a liquid scintillation counter. Aliquots were taken from half the samples and protein measured by the method of Lowry et al. (1951), allowing the determination of milligrams of protein per 1,000 3H counts and the calculation of milligrams of protein per disk. After correction for decay of 42K⁺, K⁺ uptake was normalized to uptake per milligram of cell protein. A zero time value was subtracted from each determination to correct for nonspecific binding of 42K⁺. This value was usually <15 nmol/mg protein.

**42K⁺ EFFLUX**
Cells grown on glass coverslips were incubated for 24 h with leucine [4,5-3H], labeled to equilibrium with 42K⁺ (5 μCi/ml, total K⁺ = 4.5 mM), rinsed once in fresh growth medium containing unlabeled K⁺, transferred to a perfusion chamber, and perfused with unlabeled growth medium at 0.98 ml/min at 37°C. The effluent was collected at 30-s intervals for 5 min, 10 ml scintillation fluid was added, and 42K⁺ was determined. The protein from each disk was solubilized and milligrams of protein per disk were determined as described for uptake studies, and 42K⁺ efflux per 30-s per milligram of cell protein was determined.

**Measurement of Changes in Beating Rate**
Cells grown on glass coverslips were placed in a Sykes-Moore chamber (Bellco Glass Inc., Vineland, NJ) that could be continuously perfused via inlet and outlet ports. The chamber was placed on the stage of an inverted phase-contrast microscope.
enclosed in a lucite box maintained at 37°C. The inlet side of the chamber was connected by polyethylene tubing to two syringe pumps to allow the cultures to be sequentially perfused by separate solutions. Perfusion at 0.98 ml/min did not disturb cell adhesion to the coverslips. A stable baseline beating rate of 140 ± 10 beats/min (SEM, n = 20) was observed for cultures of hearts 10 d in ovo. Beating was determined visually or by monitoring the movement of the border of a single cardiac cell with a video-motion detector and recording the output on a physiological recorder (Hewlett-Packard Co., Palo Alto, CA) as previously described (Biedert et al., 1979).

**Computer Analysis**

Data were fitted to equations describing a given binding model using a derivative-free nonlinear regression analysis described by Dixon and Brown (1977).

**RESULTS**

**Alterations in K⁺ Permeability in Heart Cell Cultures in Response to Muscarinic Agonists**

To correlate agonist-mediated decreases in muscarinic receptor number in embryonic heart cell cultures with the ability of muscarinic agonists to alter beating rate and K⁺ permeability, it was first necessary to determine the kinetics and concentration dependence of the effects of muscarinic agonists on these responses in cells not subjected to prior agonist exposure. Changes in the rate of efflux of ⁴²K⁺ after exposure of cells to muscarinic agonists were determined as a measure of the change in permeability of the cell to K⁺. To study K⁺ efflux, the intracellular K⁺ pool was first labeled to equilibrium with ⁴²K⁺. An experiment to determine the time course over which the intracellular pool equilibrated with ⁴²K⁺ added to the extracellular medium is shown in Fig. 1. Uptake of ⁴²K⁺ was half-maximal at 13.2 min and equilibration with the medium was complete at 980 ± 15 nmol/mg cell protein (SEM, n = 3) at 3 h.

Efflux of ⁴²K⁺ from control cells equilibrated with ⁴²K⁺ was found to follow an exponential time course for up to 30 min during which ⁴²K⁺ remaining in the cells decreased from 960 ± 10 nmol/mg protein (n = 3) to 285 ± 15 nmol/mg protein (data not shown). The half-time of efflux was 14 min. However, because we had demonstrated that exposure to carbamylcholine, a muscarinic agonist, for times longer than 10 min caused a decrease in the number of [³H]MS binding sites on intact cells (see below) we were concerned that measurements of the effect of carbamylcholine on rate of ⁴²K⁺ efflux for ≥10 min might be complicated by simultaneous carbamylcholine-mediated decreases in the ability of further exposure to carbamylcholine to increase ⁴²K⁺ efflux rate. For this reason, the effect of carbamylcholine on the rate of ⁴²K⁺ efflux was studied during a 5-min exposure to the agonist.

The effect of the cholinergic agonist carbamylcholine on the rate of efflux of K⁺ from cultured cells is shown in Fig. 2. Each plot in Fig. 2 represents the least-squares fit of a semilogarithmic plot of the data to a straight line. These data indicate that during the times studied, the efflux of ⁴²K⁺ follows an exponential time course and increases with increasing concentrations of car-
bamylcholine. The half-times for efflux derived from the slopes of the curves in Fig. 2 are plotted in the insert. The half-time for efflux of $^{42}\text{K}^+$ decreased 32% from 13.2 ± 0.3 min (SEM, $n = 14$) to 9.0 ± 0.2 min ($n = 14$) as the concentration of carbamylcholine varied from 0 to $10^{-3}$ M. The increase in the rate of efflux of $^{42}\text{K}^+$ from the cells over this concentration range was half-maximal at a carbamylcholine concentration of $8 \times 10^{-5}$ M. Incubation of cells with $10^{-3}$ M carbamylcholine in the presence of $10^{-6}$ M atropine completely inhibited the increase in the $\text{K}^+$ efflux rate (Fig. 2), which indicates that these effects on $\text{K}^+$ movement were specific for interaction with muscarinic receptors.

![Figure 1](https://i.imgur.com/3.png)

**Figure 1.** Equilibration of $^{42}\text{K}^+$ with the intracellular $\text{K}^+$ pool in cultured heart cells. Cultured heart cells from chick embryo hearts 10 d in ovo were grown on glass coverslips. Replicate cultures were labeled overnight in 0.2 µCi/ml leucine [4,5-3H], incubated in fresh growth medium containing 5 µCi/ml $^{42}\text{K}^+$ for the times indicated (total $\text{K}^+ = 4.5$ mM), washed and $^{42}\text{K}^+$ uptake per mg protein determined as described in Methods. Each point represents the mean of three replicate determinations and is corrected for nonspecific binding.

_Uptake of $^{42}\text{K}^+$ into Cultured Heart Cells in the Presence of Muscarinic Agonists_

The effect of carbamylcholine on the rate of uptake of $^{42}\text{K}^+$ into cultured heart cells is shown in Fig. 3. The data are reported as $^{42}\text{K}^+_{eq} - ^{42}\text{K}^+_t$, where $^{42}\text{K}^+_{eq}$ is the $^{42}\text{K}^+$ content of the cell after a 3-h equilibration with $^{42}\text{K}^+$-containing medium and $^{42}\text{K}^+_t$ is $^{42}\text{K}^+$ uptake at time $t$. Data are plotted on a logarithmic scale. This analysis allows comparison of the data from efflux and uptake studies. The time course of $^{42}\text{K}^+$ uptake was exponential over the times studied, with a half-time under control conditions of 13.8 ± 0.5 min (SEM, $n = 21$), in close agreement with the value of 13.2 ± 0.3 min derived from the uptake data in Fig. 1. The half-time of uptake in the presence of $10^{-3}$ M carbamylcholine was 8.8 ± 0.4 min ($n = 21$), similar to the value of 9.0 ± 0.2 min obtained for the half-time of efflux of $^{42}\text{K}^+$ at $10^{-3}$ M carbamylcholine in the experiments described in Fig. 2. Hence, the rates of efflux and uptake of
FIGURE 2. Effect of carbamylcholine on $^{42}$K$^+$ efflux from cultured heart cells. Dishes containing heart cells cultured from chicken embryos 10 d in ovo and grown on glass coverslips were labeled overnight with 0.2 $\mu$Ci/ml leucine [4,5-$^3$H], incubated for 3 h in growth medium 5 $\mu$Ci/ml in $^{42}$K$^+$ (total K$^+$ = 4.5 mM), rinsed, and placed in an efflux chamber as described in Methods. Cells were perfused with growth medium containing unlabeled K$^+$ and the indicated concentrations of carbamylcholine; O, no carbamylcholine; ⃝, $10^{-5}$ M; □, $10^{-4}$ M; △, $10^{-3}$ M carbamylcholine plus $10^{-6}$ M atropine. $^{42}$K$^+$ efflux was determined at the times indicated as described in Methods and plotted as $^{42}$K$^+$ remaining in the cells at time $t$, calculated by subtracting total efflux of $^{42}$K$^+$ from the initial $^{42}$K$^+$ content of the cell at time zero. $^{42}$K$^+$ at time zero was determined as the mean K$^+$ content/mg protein of 14 coverslips exposed to 5 $\mu$Ci/ml $^{42}$K$^+$ for 3 h and rinsed, and $^{42}$K$^+$ was determined as described in Methods. Each curve is the mean of two sets of seven replicate determinations each. The lines are the least-squares fit of the log of $^{42}$K$^+$ remaining at time $t$ to a straight line and are plotted on a logarithmic scale vs. time. Correlation coefficients were at least 0.99 for all plots. Insert: The effect of increasing carbamylcholine concentration on the half-time of $^{42}$K$^+$ efflux. The half-time of efflux is calculated from the relationship $T_{1/2} = \ln 2/k_1$, where $k_1$ is the slope of the curve at each carbamylcholine concentration in Fig. 2 and equals the rate constant for the movement of $^{42}$K$^+$ out of the cell.
$^{42}$K$^+$ are essentially equal, as would be expected in the steady state (see below), and respond similarly to carbamylcholine.

$K^+$ Content of Cultured Heart Cells during Incubation with Carbamylcholine

The efflux rate of K$^+$ is a function of membrane potential ($E_m$), K$^+$ gradient across the cell membrane, and permeability of the membrane to K$^+$ (Goldman, 1943). The concentration gradient of K$^+$ across the cell membrane is a major driving force in the movement of K$^+$ in response to changes in membrane permeability. If muscarinic agonists increase the permeability of the cell membrane to K$^+$, prolonged exposure to muscarinic agonists might result in depletion of intracellular K$^+$ if changes in membrane potential and pump mechanisms were unable to balance the accelerated K$^+$ loss. If the intracellular
K⁺ concentration varied with time of exposure to muscarinic agonist, the driving force for K⁺ efflux would vary and the comparison of relative efflux rates could not be used as an indicator of changes in K⁺ permeability in cells exposed to agonists for various times. Therefore, the effect of muscarinic agonists on steady state K⁺ content was measured in cells in which the intracellular K⁺ pool had been equilibrated with 5 μCi/ml ⁴²K⁺, followed by incubation for various times in ⁴²K⁺ plus 10⁻³ M carbamylcholine. A mean value of 980 ± 20 nmol/mg (SEM, n = 21) protein was obtained for control cells and values of 960 ± 30 (n = 14), 973 ± 15 (n = 14), 975 ± 25 (n = 14), and 980 ± 15 (n = 14) nmol/mg protein were obtained for cells incubated in 10⁻³ M carbamylcholine for 15, 45, 90 min, and 3 h, respectively. These values were not significantly different from control. Hence, changes in K⁺ influx and efflux rates after incubation with carbamylcholine cannot be explained by alterations in intracellular K⁺ content and are consistent with changes in K⁺ permeability.

**Binding of Muscarinic Antagonist to Intact Cultured Heart Cells**

The effects of muscarinic agonists on K⁺ permeability and beating rate require the interaction of agonist with receptors on the surface of the intact cell. Because receptor number measured in heart cell homogenates may not reflect the subset of receptors in the intact cell available for agonist binding, correlations between changes in receptor number and functional response to the agonist might be studied with greater validity using measurements of receptor number in the intact cell. Because acetylcholine, the physiologic muscarinic agonist, is a relatively hydrophilic ligand, we chose the more hydrophilic muscarinic antagonist [³H]MS for measurement of receptor number in the intact cell. We felt that the more hydrophobic antagonist [³H]QNB used in previous studies of ligand binding to whole cell homogenates (Galper and Smith, 1978; Galper and Smith, 1980) might diffuse readily through the cell membrane and bind to receptors that were not available for interaction with the agonist at the cell surface. The specific binding of [³H]MS was saturable at 195 ± 8 fmol/mg protein (SEM, n = 6) with a Kᵣ of 0.43 ± 0.05 nM (Fig. 4). 50% of bound counts were specific as measured by displacement by 10⁻⁴ M oxotremorine, a potent muscarinic agonist.

**Agonist-induced Loss of [³H]MS Binding Sites Assayed in Intact Cells: Dependence on Agonist Concentration during Pre-exposure**

We have demonstrated previously that a 3-h incubation of heart cell cultures with muscarinic agonists caused a 70% decrease in the binding of [³H]QNB to heart cell homogenates with a half-maximal effect of 35% for carbamylcholine at 0.8 × 10⁻⁵ M (Galper and Smith, 1980). When the effect of prior exposure of cells to various concentrations of agonist on receptor number was determined by binding [³H]MS directly to intact cells (Fig. 5), exposure to carbamylcholine caused a maximum decrease of 67% in specific [³H]MS binding with a half-maximal effect of 34% at 3 × 10⁻⁵ M. Hence, total receptor loss was the same as that assayed in homogenates. The somewhat higher concentration of agonist required for the loss of 50% of the receptors in
the intact cell assay might reflect a degree of reversibility of receptor loss after removal of agonist and during incubation of the metabolically intact cell with [³H]MS, which cannot take place in cell homogenates.

One alternative explanation for the loss of [³H]MS binding sites after incubation with agonist is that agonist exposure mediates a decrease in the affinity of the receptor for antagonist and hence an apparent decrease of [³H]MS binding. To rule out this possibility, an experiment comparing the concentration dependence of the binding of [³H]MS to control cells with binding to cells after prior agonist exposure is illustrated in Fig. 4. Scatchard analysis of the data gave two nearly parallel straight lines for carbamylcholine-treated and control cells, corresponding to $K_d$ values of $0.45 \pm 0.07$ (SEM, $n = 6$) and $0.43 \pm 0.05$ ($n = 3$), respectively, and intersecting the x axis at 68 fmol/mg of protein for carbamylcholine-treated cells and 195 fmol/mg of protein for control cells. Hence, these data are consistent with a 65% carbamylcholine-mediated decrease in [³H]MS binding with no significant effect on affinity for [³H]MS. Because of the low level of binding of [³H]MS to agonist-treated cells, these data were subject to somewhat more scatter than in control cells.

![Figure 4. Scatchard plot of binding of [³H]MS to chick heart cell homogenates at various [³H]MS concentrations. Replicate cultures in multiwell dishes were incubated overnight in medium containing 0.01 μCi/ml leucine [¹⁴C(U)]. Growth medium was replaced by fresh medium with and without 10⁻¹⁰ M carbamylcholine, incubated for 3 h at 37°C, and washed twice with 3 ml each of HEPES-buffered M-199 and incubated for 2 h in the presence of the indicated concentrations of [³H]MS. Cells were washed three times with 3 ml of ice-cold wash solution and specific [³H]MS binding was determined as described in Methods. Each point represents the mean of four replicate determinations repeated n times and is corrected for nonspecific binding; ●, control cells ($n = 6$); ○, cells incubated before [³H]MS binding for 3 h with 10⁻¹⁰ M carbamylcholine ($n = 3$).]
Time Course of Agonist-induced Loss of [3H]MS Binding Sites Assayed in Intact Cells

We have demonstrated previously that incubation of heart cell cultures with muscarinic agonists caused a 70% decrease in the binding of [3H]QNB to heart cell homogenates. The response was biphasic with an early rapid loss of 26% of binding sites followed by a lag phase and the gradual loss of another 43% of receptors over 2.5 h. The half-time of this second phase of loss of [3H]QNB binding sites was 30 min. Recovery of receptor sites required protein synthesis and took place over 12 h after removal of agonist (Galper and Smith, 1980).

Fig. 5. Effect of prior exposure of cells to graded concentrations of agonist on [3H]MS binding to intact cells. Replicate cultures in multiwell dishes were incubated overnight in medium containing 0.01 μCi/ml leucine [14C(U)]. Growth medium was replaced by fresh medium containing the indicated concentration of carbamylcholine, incubated for 3 h at 37°C, washed twice with 3 ml each of HEPES-buffered M-199, and incubated for 1 h in HEPES-buffered M-199 in the presence of 2 nM [3H]MS. Cells were washed three times with 3 ml of ice-cold wash solution and specific binding of [3H]MS determined as described in Methods. Each point represents the mean of four replicate determinations and is corrected for nonspecific binding.

Fig. 6 summarizes data from an experiment in which we studied the number of specific [3H]MS binding sites in the intact cell after exposure to carbamylcholine for various times. After a brief lag phase, the loss of 72% of [3H]MS binding sites in the intact cell took place during a 6-h exposure to agonists. The loss of approximately half these receptors took place over the initial 30 min. These binding sites recovered during a 12-h incubation in fresh medium. Recovery was inhibited by 2 μg/ml cycloheximide (data not shown). Hence, the time course of agonist-induced receptor loss as measured in the intact cell bears substantial similarity to the slow phase of receptor loss seen in assays carried out in homogenates (Galper and Smith, 1980). However, the rapid
loss of 26% of receptors after brief exposure of cells to agonists did not occur in the intact cell.

Computer modeling of the data in Fig. 6 attempted to fit the data for the decrease of $[\text{H}]MS$ binding sites by a nonlinear least-squares analysis to one of three models: (a) decay by a single exponential process; (b) decay by a single exponential plus a constant fraction of sites unaffected by agonist exposure; or (c) decay of two independent classes of receptors represented by two exponential functions. The best fit of the data, as indicated by the residual sum of squares and the standard deviation of estimated constants, was to the equation $A = A_0 e^{-kt} + C$, corresponding to a single exponential plus a constant fraction unaffected by agonist. Here $A$ represents total $[\text{H}]MS$ binding sites at time $t$. $A_0$, the initial number of receptors subject to agonist-mediated loss, was $112 \pm 7 \text{ fmol/mg}$; $k$, the rate constant for agonist-mediated receptor loss, was $0.019 \pm 0.003 \text{ min}^{-1}$. $C$, a constant that represents a subclass of receptors that does not respond to agonist-mediated receptor loss, was $48.6 \pm 6 \text{ fmol/mg}$ or $30\%$ of total $[\text{H}]MS$ binding sites. This analysis does not exclude the possibility of a biphasic process, but demonstrates a less satisfactory statistical fit for such a model.

**Effect of Prior Exposure to Agonist on the Ability of Muscarinic Agonists to Alter $K^+$ Permeability and Beating Rate**

We compared the effect of carbamylcholine on $K^+$ permeability and beating rate in control cells and in cells exposed for various times to $10^{-3} \text{ M carba-
mycholine (Fig. 7; Table 1). The results shown in Fig. 7 demonstrate that in
cells not previously exposed to agonist, carbamylcholine decreased the half-
time for efflux of $^{42}$K$^+$ from 13.4 ± 0.2 min ($n = 21$) to 8.8 ± 0.2 min ($n = 21$),
a decrease of 4.6 min or 33% below control. However, after a 3-h exposure to

10$^{-3}$ M carbamylcholine, subsequent exposure to carbamylcholine had no
significant effect on rate of $^{42}$K$^+$ efflux.

Analysis of the time course over which carbamylcholine exposure decreased
the response of K$^+$ efflux to muscarinic agonists is shown in Table I. During
the first 15 min of agonist exposure, no significant effect on the muscarinic response could be detected; carbamylcholine decreased the half-time for efflux by 4.4 min, from 13.2 min to 9.0 min, compared with a 4.6-min decrease for control cells. However, after a 30-min agonist exposure, carbamylcholine decreased efflux half-time by only 2.3 min, from 13.2 min to 10.9 min, a loss of 52% of the effect seen in control cells. After a 45-min agonist exposure, carbamylcholine decreased efflux half-time by only 1.3 min, from 13.3 min to 12.0 min, a loss of 74% of the effect seen in control cells. After a 90-min exposure to agonist, carbamylcholine had no measureable effect on efflux rate. Because the recovery of binding sites after agonist-mediated receptor loss takes place during a 12-h incubation in fresh medium, studies were carried out to determine the ability of agonist to mediate an increase in K⁺ efflux in cells subjected to agonist pre-exposure followed by recovery in fresh medium. In the experiments summarized in Table I, such a 12-h recovery period was accompanied by full restoration of the K⁺ permeability response.

We also determined the effect of prior exposure to carbamylcholine on the ability of muscarinic agonists to slow beating rate in cultured heart cells. As
summarized in Table II, in control cells $10^{-3}$ M carbamylcholine decreased beating rate by 15% from $140 \pm 3$ beats/min (SEM, n = 20) to $119 \pm 3$ beats/min. A 15-min prior exposure to agonist had no effect on the beating rate response to carbamylcholine. However, after a 30-min prior exposure to agonist, carbamylcholine decreased beating rate by only 8% to $129 \pm 2$ beats/min, a response only 47% of that in control cells. A 45-min prior exposure to agonist decreased the response of beating rate to 4% ($136 \pm 1.4$ beats/min), which did not represent a statistically significant difference from the control rate of $140 \pm 3$ beats/min. Finally, after a 180-min prior exposure to agonist, no response in beating rate to carbamylcholine could be detected. However,

**TABLE II**

<table>
<thead>
<tr>
<th>Duration of pre-exposure to agonist</th>
<th>Percent decrease in beating rate (±SEM, n = 20)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>$15\pm2$</td>
</tr>
<tr>
<td>15 min</td>
<td>$15\pm1$</td>
</tr>
<tr>
<td>30 min</td>
<td>$8\pm1$</td>
</tr>
<tr>
<td>45 min</td>
<td>$4\pm1$</td>
</tr>
<tr>
<td>180 min</td>
<td>$0\pm2$</td>
</tr>
<tr>
<td>180 + 12 h recovery</td>
<td>$15\pm1$</td>
</tr>
</tbody>
</table>

* Cells grown on glass coverslips were perfused in Sykes-Moore chambers and the beating rate was determined as described in Methods. For each determination cells were first perfused with growth medium. After establishment of a stable baseline, perfusion was continued with medium $10^{-3}$ M in carbamylcholine for the times indicated, after which cells were perfused with fresh medium for 5 min and the effect of reperfusion with $10^{-3}$ M carbamylcholine on beating rate was determined. All observations in a given series were performed on the same group of cells in the microscope field.

‡ Difference in the decrease in beating rate between 15 and 30 min of agonist pre-exposure was significant with $P < 0.001$, and the difference between 30 and 45 min of pre-exposure was significant at $P < 0.01$, whereas the difference between 45 min and 180 min of pre-exposure was not statistically significant.

if these cells were washed and incubated for 12 h in fresh medium, the beating rate in these cells became fully responsive to carbamylcholine.

**Correlation between Agonist-induced Changes in Receptor Number and the Ability of Muscarinic Agonists to Alter K⁺ Permeability and Beating Rate**

Analysis of the time dependence of agonist-induced decrease in receptor number (Fig. 6) demonstrated two subclasses of $[^3]$H]MS binding sites: a subclass of 70% of receptors that were lost with a half-time of 30 min and a subclass of 30% of receptors whose binding of $[^3]$H]MS was unaffected by up to 6 h of exposure to agonist. After a 90- or 180-min pre-exposure to agonist, no K⁺ efflux rate or beating rate responses to agonist could be demonstrated (Tables I and II). However, 30% of receptor sites were still available for
antagonist binding (Fig. 6). One explanation of these data is that this 30% of sites alone is not sufficient to elicit a cellular response and that the fraction of receptors subject to agonist control may play a critical role in mediating K⁺ permeability and beating rate responses. The correlation between the time course of the agonist-mediated decrease in the binding of [³H]MS to the subclass of receptors subject to agonist control and the time course of the decrease in K⁺ permeability and beating rate response is shown in Fig. 8. A close correlation between the decrease in the response in K⁺ permeability and beating rate was observed. Such a correlation is consistent with the view that the increase in K⁺ permeability occurs in parallel with and is related to the decrease in beating rate. Agonist-mediated changes in receptor number also closely approximated decreases in functional response. A 15-min lag period was followed by a decrease in all three parameters, which was maximal after 6 h with a half-maximal effect at 30 min.

Effect of Agonist Concentration during Pre-exposure on Response of K⁺ Permeability to Carbamylcholine

The effect of a 3-h exposure of cells to various concentrations of carbamylcholine on the ability of carbamylcholine to decrease the $T_{1/2}$ of K⁺ efflux is summarized in Table III. The effect was half-maximal at $3 \times 10^{-5}$ M carbamylcholine, with no measurable effect at concentrations below $3 \times 10^{-6}$
The correlation between the data in Table III and the concentration dependence of agonist-induced receptor loss as measured by \[^3H\]MS binding in the intact cell is shown in Fig. 9. In this series of experiments, carbamylcholine caused a maximum decrease of 69% in specific \[^3H\]MS binding to intact cells. A half-maximal effect of 34% was seen at \(3.0 \times 10^{-5}\) M carbamylcholine. The percent decrease in \[^3H\]MS binding to the subclass of 69% of receptors subject to agonist control demonstrates a good correlation with the decrease in the ability of carbamylcholine to decrease the half-time for \(K^+\) efflux. However, a given decrease in receptor sites was associated with a somewhat larger decrease in the \(K^+\) efflux rate response. A similar discrepancy between \(\beta\)-adrenergic antagonist binding and adenylate cyclase activation was seen after agonist-induced decreases in \(\beta\)-adrenergic receptor number in frog erythrocytes (Wessels et al., 1978).

Although these data suggest that receptor number is the critical factor controlling responsiveness to muscarinic agonists, another possible explanation for the agonist-mediated decrease in \(K^+\) permeability response is that prior exposure to agonist might decrease receptor affinity for agonist. If this were the case, however, perfusion of cells during efflux studies with higher concentrations of agonists should compensate for such an apparent decrease in responsiveness. An experiment summarized in Table IV demonstrates that in

### Table III

EFFECT OF PRE-EXPOSURE TO VARIOUS CONCENTRATIONS OF AGONIST ON THE ABILITY OF AGONIST TO DECREASE THE \(T_{1/2}\) OF \(K^+\) EFFLUX

<table>
<thead>
<tr>
<th>Concentration of carbamylcholine during pre-exposure</th>
<th>Control</th>
<th>Carb</th>
<th>Decrease in response to muscarinic agonist (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M)</td>
<td>(T_{1/2}) of (K^+) efflux (± SEM; (n = 7))*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.4±0.2</td>
<td>8.5±0.2</td>
<td>0</td>
</tr>
<tr>
<td>(1 \times 10^{-6})</td>
<td>13.2±0.1</td>
<td>8.5±0.2</td>
<td>0</td>
</tr>
<tr>
<td>(3 \times 10^{-6})</td>
<td>13.4±0.3</td>
<td>9.2±0.2§</td>
<td>15</td>
</tr>
<tr>
<td>(1 \times 10^{-5})</td>
<td>13.5±0.3</td>
<td>9.9±0.2§</td>
<td>30</td>
</tr>
<tr>
<td>(3 \times 10^{-5})</td>
<td>13.5±0.3</td>
<td>11.4±0.3§</td>
<td>61</td>
</tr>
<tr>
<td>(1 \times 10^{-4})</td>
<td>13.5±0.2</td>
<td>12.2±0.2§</td>
<td>76</td>
</tr>
<tr>
<td>(1 \times 10^{-3})</td>
<td>13.2±0.2</td>
<td>13.5±0.2§</td>
<td>100</td>
</tr>
</tbody>
</table>

* Cells grown on glass coverslips were labeled overnight with leucine [4,5-\(^3H\)] (0.2 μCi/ml), after which cells were incubated at 37°C in fresh growth medium containing the indicated concentrations of carbamylcholine plus 5 μCi/ml \(^4K^+\) (K = 4.6 mM). Cells were rinsed and \(K^+\) efflux was determined under control conditions or in the presence of \(10^{-2}\) M carbamylcholine as described in Methods. Half-times of efflux were derived from least-squares analysis of the data as described in Fig. 2. Correlation coefficients were at least 0.99 for all plots. Steady state intracellular \(K^+\) contents in all experiments were not significantly different from the control value of 970 ± 25 nmol/mg.

§ Differences between successive measurements of half-time for efflux in the presence of carbamylcholine are significant within 99% confidence limits as determined by the method of covariance analysis (Dixon and Massey, 1957).

‡ Calculated from the decrease in \(T_{1/2}\) of \(K^+\) efflux due to carbamylcholine exposure as a percent decrease in the maximal effect of carbamylcholine on \(T_{1/2}\) of efflux seen in cells not subjected to agonist pre-exposure.
cells exposed for 30 min to $10^{-3}$ M carbamylcholine, the ability of perfusion with $10^{-3}$ M carbamylcholine to shorten the $T_{1/2}$ of K$^+$ efflux decreased by 50% from 4.5 min (14.1–9.6 min) in control cells to 2.3 min (13.9–11.6 min) in cells pre-exposed for 30 min to agonist. Increasing the concentration of carbamylcholine in the perfusate to $5 \times 10^{-3}$ M did not produce a significant decrease in $T_{1/2}$ of efflux in either control cells or in agonist-pretreated cells. These data strongly suggest that the response of both control cells and agonist-pretreated cells was already maximal at $10^{-3}$ M carbamylcholine and that decreased responsiveness caused by agonist pre-exposure was not the result of an agonist-mediated decrease in receptor affinity for agonist.

**DISCUSSION**

The classical explanation for the effect of acetylcholine on the mechanical and electrical activity of atrial muscle has been based on the finding that acetylcholine selectively increases the membrane permeability to potassium...
ions (Hutter, 1961). More recently, acetylcholine has also been shown to decrease the slow inward calcium current, $I_{Ca}$, in atrial tissue (Giles and Noble, 1976; Ten Eick et al., 1976). This decrease in $Ca^{2+}$ entry into the cell would contribute to the shortening of the action potential duration and decrease in the force of contraction. Recent studies in guinea pig heart have demonstrated that in ventricular tissue, acetylcholine decreases $I_{Ca}$ and the time-dependent outward current, but has no effect on the time-independent, inwardly rectifying outward K$^+$ current (Hino and Ochi, 1980). In the studies of cultured chick embryo heart cells reported here, the close parallel between the small but reproducible carbamylcholine-induced decrease in beating rate and the carbamylcholine-mediated decrease in the half-time of K$^+$ efflux (Fig. 8) supports the view that, in these cells, increases in K$^+$ permeability may be responsible, at least in part, for the observed decrease in the rate of beating. It should be noted in this context that although we consider it highly probable that the increased K$^+$ fluxes in response to carbamylcholine reflect increased K$^+$ permeability, Garnier et al. (1976) demonstrated that in frog atrial trabeculae the current induced by acetylcholine ($I_{Ach}$) shows inward-going rectification at membrane potentials ~25–30 mV positive to the resting potential. In the range of inward-going rectification, changes in membrane potential and permeability would not be directly related to changes in current flow or changes in the rate of K$^+$ efflux. It is uncertain whether the cultured chick embryo heart cells studied here have similar inward-going rectification properties. However, our K$^+$ efflux measurements were carried out on beating cells, the resting membrane potentials of which average ~70 to ~60 mV (J. Hasin, unpublished observations), appreciably lower than the reported region of inward-going rectification for $I_{Ach}$. In addition, in the presence of carba-

<table>
<thead>
<tr>
<th>Concentration of carbamylcholine in perfusate</th>
<th>$T_{1/2}$ of $^{42}$K$^+$ efflux (± SEM, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M$</td>
<td>min</td>
</tr>
<tr>
<td>0</td>
<td>14.1±0.2</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>9.6±0.4‡</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$</td>
<td>8.9±0.2‡</td>
</tr>
</tbody>
</table>

* Cells grown on glass coverslips were labeled overnight with leucine [4,5-3H] (0.2 μCi/ml). Cells were incubated for 2.5 h at 37°C in fresh growth medium containing 5 μCi/ml $^{42}$K$^+$ (total K$^+ = 4.8$ mM), after which coverslips were incubated for an additional 30 min in medium with or without $10^{-3}$ M carbamylcholine. Cells were rinsed and K$^+$ efflux was determined as described in Methods in the presence of the indicated concentrations of carbamylcholine in the perfusate. Half-times of efflux were derived from least-squares analysis of the data. Correlation coefficients were at least 0.99 for all plots. Steady state intracellular K$^+$ contents in all experiments were not significantly different from the control value of 950 ± 30 nmol/mg protein.
‡ Differences between measurements of half-time for efflux at successive carbamylcholine concentrations were not statistically significant.
mylcholine it is likely that these cells are hyperpolarized. Thus, although it cannot be stated with certainty, these data taken together suggest that the observed enhancement for K⁺ efflux reflects increased K⁺ permeability in response to carbamylcholine.

If the rate of K⁺ efflux is expressed as a fraction of total ⁴²K⁺ released per minute, the rate of efflux under control conditions can be compared with values determined by other workers. Such a comparison places the efflux rate reported here, 5%/min, intermediate between that reported in growth-oriented chick heart cell cultures (10%/min; Horres and Lieberman, 1977), and the value of 3.5%/min derived from efflux data from strips of chick ventricle from embryos 6–8 d in ovo (Carmeliet et al., 1975). It should be noted that studies reported here were carried out on confluent monolayers beating at a rate of 140 ± 4 beats/min, whereas growth-oriented chick heart cell cultures grown on thin nylon filaments were electrically paced at 150 beats/min; chick ventricle strips, representing a thin section of intact tissue, were presumably quiescent. Differences in cell geometry, beating rate, and the state of aggregation of cells in culture would be expected to be associated with differences in ion flux rates and in electrical properties of cardiac tissue, and may account for the differences observed in the rate of K⁺ efflux in these three systems.

If the efflux of K⁺ is dependent on the beating rate, comparisons between rates of K⁺ efflux in control cells and cells exposed to muscarinic agonists in which the beating rate is decreased 15% (Table II) would tend to underestimate the effect of muscarinic agonists on K⁺ permeability. The data for K⁺ efflux presented here have not been corrected for these effects of muscarinic agonists on the beating rate.

The relative insensitivity of these cells to carbamylcholine is of concern. Comparison of the sensitivity of heart cell cultures and intact hearts to muscarinic agonists in prior studies has demonstrated a marked decrease in sensitivity of beating rate of cultured cells to muscarinic agonists. The half-maximal concentration of carbamylcholine for inhibition of beating in intact chick embryo hearts removed from the embryos 7–10 d in ovo was 10 nM (Galper et al., 1977). In the present study, a 15% decrease in beating rate was seen within 10–15 s after exposure of cultures of hearts taken from embryos 10 d in ovo to 10⁻³ M carbamylcholine. Sporalakis and Lehmkuhl (1965) reported that heart cell cultures were insensitive to muscarinic agonists; Ertel et al. (1971) observed a response only after a 15-min exposure to agonist, with a decrease in beating rate that was independent of agonist concentration. A third group reported a response to as little as 1 nM acetylcholine using a rapid flow technique (Hermnsmeyer and Robinson, 1977).

The limited sensitivity of our cultures to muscarinic agonists may be related to several aspects peculiar to cultured systems. One factor is predominance in our cultures of ventricular cells, which are relatively insensitive to muscarinic stimulation.

Josephson and Sporalakis (1982) demonstrated that acetylcholine produced a decrease in J₄₄ in aggregates of embryonic chick heart only in the presence of β-adrenergic stimulation. Watanabe et al. (1978) and Jakobs et al. (1979) demonstrated that muscarinic agonists inhibit β-adrenergic agonist-stimulated
adenylate cyclase activity. The resulting decrease in cyclic AMP levels could contribute to the effect of muscarinic agonists on the intact heart. Although intact hearts are subject to some degree of endogenous β-adrenergic stimulation, the cultures of chick embryo heart cells studied here are devoid of such effects. Hence, muscarinic agonists could only affect adenylate cyclase activity in cultured heart cells by lowering basal adenylate cyclase activity. Biegon and Pappano (1980) observed that in embryonic chick ventricles studied before hatching, carbamylcholine decreased β-adrenergic agonist-stimulated cyclic AMP levels, but had little effect on basal cyclic AMP levels. Although other mechanisms probably account in part for the modest muscarinic effects in these cultures, the absence of significant β-adrenergic stimulation in cultured cells would be expected to contribute to the limited responsiveness of these cells to muscarinic agonists.

In the present studies the rate of K⁺ efflux was increased by 33% in the presence of 10⁻³ M carbamylcholine. In the absence of other compensatory mechanisms, the increase in K⁺ flow down its concentration gradient would tend to deplete intracellular K⁺ ([K⁺]ᵢ). Under these conditions the interpretation of changes in the rate of K⁺ efflux would be complicated because they would represent an increase in permeability to K⁺ at the same time that the driving force for movement of K⁺ out of the cell was decreasing. However, in the studies reported here, the undirectional rates of efflux and uptake of ⁴²K⁺ are equal from 30 s until 5 min after exposure to agonist. Furthermore, exposure of cells to agonist for time periods from 15 min to 6 h had no effect on [K⁺]ᵢ. The absence of any effect of prolonged agonist exposure on [K⁺]ᵢ could potentially be explained by the absence of an effect of muscarinic agonists on K⁺ permeability in cells after agonist exposures of 90 min or longer (Table I). However, agonist exposures of 15–45 min are associated with a significant increase of K⁺ permeability (Table I) and in these cells a compensatory mechanism for maintenance of [K⁺]ᵢ at control levels must exist. Hence, the passive movement of K⁺ down its concentration gradient is presumably balanced by an increase in the passive movement of K⁺ into the cell caused by the muscarinic agonist-induced hyperpolarization of the cell membrane, by an increase in active transport of K⁺ into the cell via NaK-ATPase (which would also tend to cause hyperpolarization), or by a combination of the two. Because efflux and influx rates for K⁺ are equal 30 s after addition of agonist, any compensatory mechanism must respond within the first several seconds of agonist exposure to maintain steady state levels of [K⁺]ᵢ. Experiments to delineate these phenomena are planned.

If K⁺ accumulated in the extracellular cleft spaces during prolonged stimulation by muscarinic agonists, then the driving force for movement of K⁺ down its concentration gradient would be decreased. Such an effect of prolonged agonist exposure on extracellular K⁺ would contribute to the decrease in the ability of subsequent exposure to muscarinic agonists to increase the rate of efflux. Cohen and Kline (1982) have recently reviewed the evidence that during repetitive electrical stimulation, [K⁺]ᵢ in the extracellular cleft spaces as measured by a K⁺-sensitive electrode in frog ventricle and several other preparations is subject to significant increases. Furthermore,
Kronhaus et al. (1979) have demonstrated a post-vagal accumulation of K+ in the rabbit sinoatrial node.

In our studies, after prolonged agonist pre-exposure, responsiveness of beating rate and rate of K+ efflux to muscarinic agonists began to recover only after a 3-h incubation in fresh medium. Recovery was complete after 12 h and required protein synthesis. If K+ were accumulating in extracellular clefts, it seems unlikely that any such K+ would require 12 h before it was fully washed away. Furthermore, work by Jalife et al. (1980) and Tokimasa et al. (1980) demonstrated that hyperpolarization of kitten sinoatrial node and bullfrog atrium were not maintained after prolonged vagal stimulation. A similar decrease in responsiveness to muscarinic agonist was seen after prolonged iontophoretic application of carbamylcholine. After washout of carbamylcholine, membrane potential returned to control levels, whereas the response to further carbamylcholine remained depressed. The authors argue that if the loss of responsiveness to carbamylcholine were caused by accumulation of extracellular K+, then after washout of carbamylcholine membrane potential would not return to normal.

To define the effectiveness with which our washing procedure removed extracellular K+, we loaded cells to asymptote with 42K+ at total extracellular K+ concentrations of 10 and 20 mM. At 10 mM K+ the maximum ability of carbamylcholine to increase K+ efflux was 50% of that seen at 4.0 mM K+, whereas at 20 mM K+ no effect of carbamylcholine on efflux rate was seen. However, if cells loaded with 42K+ in the presence of high extracellular K+ were washed in a solution containing 4.6 mM K+ (at the same specific activity of 42K+) for 15 min, the efflux rate returned to within 15% of control values. These observations argue strongly that high concentrations of K+ can be washed out by brief periods of exposure to a solution containing physiologic concentrations of K+, and hence delayed K+ washout is unlikely to account for the loss of responsiveness to carbamylcholine seen in cells preexposed to agonists.

Comparison of the agonist-induced loss of receptors as measured in the intact cell (Fig. 5) with the slow phase of agonist-induced receptor loss measured in homogenates of intact cells that had been exposed to agonist before homogenization (Galper and Smith, 1980) reveals substantial similarities. Both assays demonstrate a lag period followed by gradual loss of binding sites. In each case, half of the slowly lost sites disappear within 30 min. In both assays, total receptor loss is ~70%, whereas 30% of receptors appear to be unaffected by agonist exposure. Receptor number recovers after 12 h in fresh medium in each case. The major difference in results obtained using the homogenate and intact cell assays is that the rapid loss of a subclass of receptors after brief agonist exposure cannot be demonstrated by the binding of [3H]MS to the intact cell. One explanation for this difference might be that after agonist exposure in the intact cell assay, cells are incubated for 1 h in the absence of agonist and in the presence of an antagonist under conditions that support normal energy metabolism. Under these conditions, functions occurring in the intact cell, but not in a homogenate, could permit recovery of a rapidly lost subset of muscarinic binding sites.
The availability of techniques for the measurement of changes in $K^+$ permeability and beating rate, together with the ability to quantify alterations in receptor number during pre-exposure to agonist, afforded us the opportunity to study the relationship of receptor number to the ability of agonist to effect a muscarinic response and to determine whether a particular subclass of receptors was critical to the mediation of such a muscarinic response. Human chorionic gonadotropin (hCG) binding to interstitial cells of the rat testis, for example, has been shown to stimulate the synthesis of testosterone and to increase levels of cyclic AMP. Low levels of $[^{125}I]$hCG sufficient to bind to only 1% of receptor sites stimulated testosterone production, and a maximum cyclic AMP response was seen at concentrations of $[^{125}I]$hCG capable of occupying only 50% of sites. Hence, for the testosterone response, 99% of receptors are not required to elicit an appreciable increase in synthesis, whereas 50% of $[^{125}I]$hCG binding sites might be classified as "spare" receptors for the cyclic AMP response (Mendelson et al., 1975).

In the present studies of the agonist-induced decrease in muscarinic receptor number, a subclass of 30% of receptors appeared to be unaffected by up to 6 h of agonist exposure. Cells exposed to agonist for 90 or 180 min, in which $[^3H]$MS binding demonstrated the presence of only this subclass of 30% of receptors, did not respond to carbamylcholine as measured by an effect on either beating rate or rate of $K^+$ efflux. Whether this subclass of 30% of receptors might mediate some as yet undefined muscarinic response, whether their presence is necessary for muscarinic activity but not sufficient to mediate a response, or whether they represent a subclass of spare receptors cannot be determined from the available data. What is clear is that the 70% of receptors subject to agonist control is critical for mediation of the muscarinic responses studied, and that there are few if any spare receptors in this subclass. Thus, agonist-induced changes in receptor number appear to modulate responsiveness to muscarinic cholinergic stimuli in cultured heart cells.

The absence of a more complete correlation of functional response and receptor number within the 70% subset could be the result of the fact that binding of $[^3H]$MS, an antagonist, may overestimate the number of physiologically active receptor sites and may not correspond on a one-to-one basis to those sites that form an active receptor complex with agonist. Comparison of the binding of radiolabeled $\beta$-adrenergic agonists and antagonists in frog erythrocyte membranes after pre-exposure to isoproterenol demonstrated that antagonist binding overestimated those receptor sites available for agonist binding (Wessels et al., 1978).

In addition to changes in the muscarinic receptor population per se, the lack of responsiveness of cultured heart cells to muscarinic stimulation after agonist pre-exposure might be caused, at least in part, by uncoupling of the formation of an agonist-receptor complex from an intermediate step critical to the mediation of a functional response. A number of cellular events have been associated with the binding of muscarinic agonists including inhibition of $\beta$-adrenergic agonist-stimulated adenylate cyclase activity (Watanabe et al., 1978), increased cyclic GMP levels (George et al., 1970), phosphorylation of specific cellular proteins by an activated protein kinase (Greengard, 1976),
and changes in phosphatidyl inositol turnover (Michell et al., 1976). Uncoupling of any of these events from agonist binding may prevent the expression of a muscarinic response.

In conclusion, critical steps in neurotransmitter-induced changes in membrane properties of excitable tissues are presumed to include the formation of an agonist-receptor complex and the coupling of that complex formation to the physiologic response. In the experiments reported here, binding of muscarinic agonist to a critical number of sites in a specific subclass of receptors was closely correlated with agonist-induced changes in unidirectional K⁺ flux and beating rate. Although the relatively high concentrations of muscarinic agonist required to produce changes in K⁺ efflux and beating rate leave uncertain the relevance of these findings to the physiologic muscarinic response, analogous mechanisms may operate in more highly sensitive intact preparations (Halvorsen and Nathanson, 1981). Our data also demonstrate the presence of a subclass of receptors that appears not to be involved in a measurable physiologic response, but there is no evidence for the existence of a large fraction of "spare" receptors. These findings support the concept that the cardiac cell is capable of modulating the level of muscarinic responsiveness through agonist-induced changes in receptor number.

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