

# Muscarinic Activation of Ionic Currents Measured by a New Whole-Cell Recording Method

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**ABSTRACT** A new method is described as an alternative to whole-cell recording in order to prevent "wash-out" of the muscarinic response to acetylcholine (ACh) in rat lacrimal gland cells. The membrane of a cell-attached patch is permeabilized by nystatin in the patch pipette, thus providing electrical continuity between the pipette and the cytoplasm of the cell without the loss or alteration of cytoplasmic compounds necessary for the maintenance of the response to ACh. With normal whole-cell recording in these cells, the response to ACh, seen as the activation of Ca-activated K and Cl currents, lasts for ~5 min. With the nystatin method, the response is not diminished after 1 h. Nystatin, applied extracellularly, is shown to cause a rapid and reversible increase of membrane conductance to cations. In the absence of wash-out, we were able to obtain dose-response curves for the effect of ACh on Ca-activated K currents. An increase of [ACh] caused an increase in the K current, with apparent saturation at concentrations above ~1  $\mu$ M ACh. The delay between ACh application and the activation of K current was inversely related to [ACh] and reached a minimum value of 0.7–1.0 s at high [ACh].

## INTRODUCTION

Tight-seal whole-cell recording (Hamill et al., 1981; Marty and Neher, 1983) has replaced microelectrode recording in many electrophysiological experiments over the last several years, especially in studies of isolated mammalian cells. Although the method has several well-known advantages, one disadvantage is a phenomenon called "wash-out," in which an ionic current disappears during the diffusional exchange between the cytoplasm and the contents of the pipette (Marty and Neher, 1983; Fernandez et al., 1984; Trautmann and Marty, 1984; Dufy et al., 1986). Wash-out could be due to the loss or alteration of enzymes or second messengers critical to the function of ion channels. Typically, wash-out occurs over a time course of minutes. Apart from decreasing the rate of exchange by selecting pipettes with small openings (Trussell and Jackson, 1987; Yakel et al., 1988; Zimmerberg, J., and A. Marty, manuscript in preparation), two methods have been used to slow or prevent wash-out. The first is to add back a cytosolic extract to the pipette solution

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(Dufy et al., 1986). In the second method (Lindau and Fernandez, 1986), called "slow whole-cell recording," a cell-attached patch of a mast cell is partially permeabilized by including ATP in the pipette solution. Extracellular ATP causes an increase in conductance in mast cells (Bennet et al., 1981). With slow whole-cell recording, the mast cells are capable of responding to an extracellular secretagogue during electrical recording. By contrast, the response to the secretagogue rapidly washed out in typical whole-cell recordings (Lindau and Fernandez, 1986). The adjective "slow" used in the name of this method refers to the capacitative currents, which have a slow time course. This is due to the series resistance, which was 100–5,000-fold higher than in typical whole-cell recordings. We have followed the idea of these experiments, but used the pore-forming antibiotic nystatin to increase the conductance of a cell-attached patch. Nystatin has two advantages over ATP. First, unlike ATP, nystatin is a pore-former that does not require receptors specific to a limited number of cells. Second, the conductance induced by nystatin in our experiments approached that seen in typical whole-cell recordings, so that high-quality voltage clamp was possible.

Using this approach, we were able to obtain, for the first time, the dose-response relationship for currents induced by acetylcholine (ACh) in rat lacrimal gland cells. These cells have muscarinic receptors that produce an electrical response through activation of a GTP-binding protein (Evans and Marty, 1986). The response, seen as the activation of three types of Ca-activated current (Marty et al., 1984), requires the release of  $\text{Ca}^{2+}$  from intracellular stores in response to an elevation of inositol trisphosphate ( $\text{IP}_3$ ), and thus depends on the integrity of a variety of cytoplasmic and membrane-bound constituents. In typical whole-cell recordings, the response to ACh washes out within ~5 min. In the experiments reported here, the response to ACh did not diminish after >1 h of recording. A preliminary report of these results has appeared (Horn et al., 1988).

#### METHODS

The experimental methods for the preparation of lacrimal gland cells and standard electrophysiology, using whole-cell recording, have been described previously (Marty et al., 1984). Briefly, cells were enzymatically dissociated from rat exorbital lacrimal glands and kept for up to 8 h in an incubator at 37°C until use. Recordings were obtained at room temperature (20–22°C) after replacement of the incubation medium by a solution containing (millimolar): 140 NaCl, 5 KCl, 1  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 5 Na-HEPES, pH 7.2. The standard internal solution contained (millimolar): 140 KCl, 0.5 EGTA, 5 K-HEPES, pH 7.2. The [EGTA] was chosen to keep the intracellular  $[\text{Ca}^{2+}]$  low while allowing intracellular  $[\text{Ca}^{2+}]$  to rise when the cells were stimulated by ACh (Marty et al., 1984). Solutions could be applied to the bath with a fast solution-exchange device (Krishtal and Pidoplichko, 1980). The exchange time was ~30 ms (see Results), provided that care was taken to place the cell close (within 30  $\mu\text{m}$ ) to the opening of the glass capillary containing the test solution (Fenwick et al., 1982). Drug removal, however, was slow, particularly after prolonged applications. No bulk bath perfusion was performed in the present experiments, so removal was mostly dependent on reaspiration of the drug into the glass capillary.

Nystatin (Sigma Chemical Co., St. Louis, MO) was freshly dissolved in methanol (5 mg/ml) and subsequently added to the internal solution at a final concentration of 20–100  $\mu\text{g}/\text{ml}$ . An ultrasonicator facilitated the solvation. Refrigerated methanol stocks were kept for up to 3 d;

final internal solutions were kept for up to 2 h. The recording pipette was coated with a thick layer of Sylgard to reduce the capacitance between the electrode and the bath. The capacitance and series resistance of the cell were monitored every 3–5 min by zeroing, with the patch-clamp amplifier (EPC-7, List-Electronic, Darmstadt, Federal Republic of Germany), the capacity transient induced by a voltage pulse. Recordings were started when the series resistance reached a value of <50 MΩ. During all recordings, series resistance compensation (70–90%) was used.

### *Properties of the Junction between the Pipette and the Cell Interior*

Nystatin forms cation-selective channels between the pipette and cytosol, as will be shown below. The permeability for Cl<sup>−</sup> is not negligible, however (see Russell et al., 1977; Kleinberg and Finkelstein, 1984), so both K<sup>+</sup> and Cl<sup>−</sup> will equilibrate within a short time (on the order of minutes) inside the cell. On the other hand, large anions in the cell are not expected to be permeant. The situation is thus essentially a Donnan equilibrium.

Let us take as an example a cell containing a concentration [P]<sub>i</sub> = 100 mM of an impermeant monovalent anion (P<sub>i</sub> stands for intracellular proteins and other large anions). The pipette solution in our experiments contained [K]<sub>p</sub> = [Cl]<sub>p</sub> = 140 mM. We designate ion concentrations within the cell by the subscript “i” and those outside the cell (i.e., in the bath) by the subscript “o.” The voltages of the pipette, cell, and bath are denoted by V<sub>p</sub>, V<sub>i</sub>, and V<sub>o</sub>, respectively. Three equations describe the equilibrium condition, assuming that the cell volume is insignificant by comparison with that of the pipette. Thus,

$$[K]_i = [Cl]_i + [P]_i, \quad (1)$$

$$[K]_i/[K]_p = \exp\{(V_p - V_i)F/RT\}, \quad (2)$$

and

$$[Cl]_i/[Cl]_p = \exp\{(V_i - V_p)F/RT\}. \quad (3)$$

Here *R*, *T*, and *F* have their usual values (i.e., at room temperature, *RT/F* ≈ 25 mV). The three equations have three unknowns. In the specific example here, the solution shows that at equilibrium [Cl]<sub>i</sub> = 99 mM, [K]<sub>i</sub> = 199 mM, and V<sub>i</sub> − V<sub>p</sub> = −8.8 mV. Note that the cell is hypertonic to both the pipette and bath. Therefore, water will enter, causing it to swell. However, we did not see obvious swelling of the cells over the period of our recordings (~60 min). Nevertheless, one possible solution to this osmotic problem is to replace some of the Cl in the pipette with an anion (e.g., SO<sub>4</sub><sup>2−</sup>) that, like some of the cytoplasmic anions, is totally impermeant through nystatin channels.

The voltage drop, V<sub>i</sub> − V<sub>p</sub>, causes a shift in the activation curve of voltage-dependent currents (e.g., see Marty and Neher, 1983). This voltage, however, has no effect on the expected reversal potentials for ion channels in the plasma membrane. Take the case, for example, of a Cl conductance. At the reversal potential, there is no Cl current across the plasma membrane, so that V<sub>i</sub> − V<sub>o</sub> = *RT/F* · log([Cl]<sub>i</sub>/[Cl]<sub>o</sub>). Thus,

$$\begin{aligned} V_p - V_o &= (V_p - V_i) + (V_i - V_o) \\ &= RT/F \{ \log([Cl]_p/[Cl]_i) + \log([Cl]_i/[Cl]_o) \} \\ &= RT/F \cdot \log([Cl]_p/[Cl]_o). \end{aligned}$$

Therefore, the reversal potential for a Cl current depends only on the Cl concentrations in the pipette and in the bath, and is independent of the intracellular Cl concentration. In the experiments reported below, [Cl]<sub>p</sub> = 140 mM and [Cl]<sub>o</sub> = 149 mM. Therefore, E<sub>Cl</sub> ≈ −1.6 mV.

## RESULTS

We initially describe a new method for measuring ACh-induced currents in lacrimal cells in the absence of wash-out. We then present dose-response curves for the effects of ACh on Ca-activated K currents.

*The Nystatin Method*

The technical challenge of our experiments was to record ACh-induced currents in the absence of wash-out (Fig. 1 *A*), which causes a complete abolition of sensitivity to

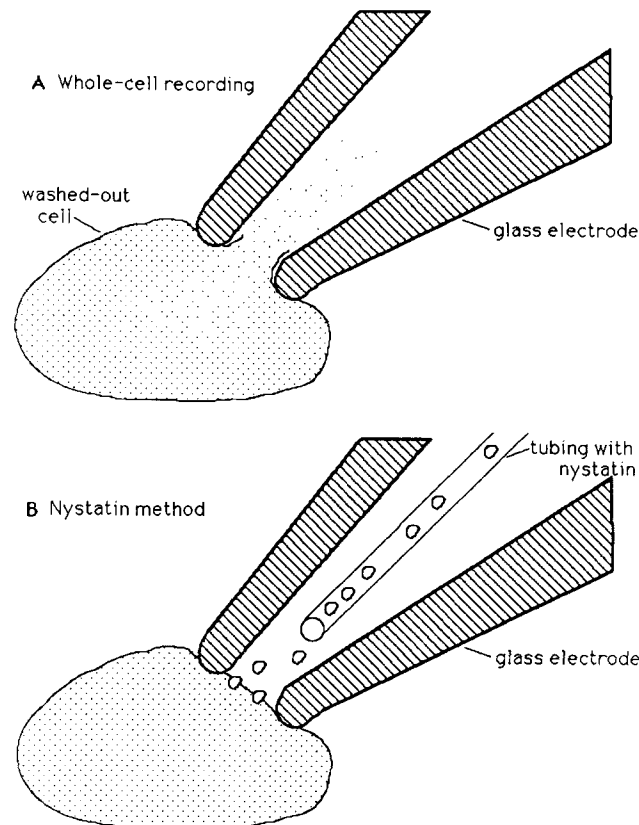


FIGURE 1. Whole-cell vs. recording with nystatin. (A) Normal whole-cell recording with wash-out. (B) The nystatin method.

ACh within ~5 min in typical whole-cell recording (Trautmann and Marty, 1984). Other experiments in this laboratory suggested that wash-out was probably the result of diffusion of molecules of 100–500 mol wt from the cytoplasm to the pipette (Zimmerberg, J., and A. Marty, unpublished observations). We therefore permeabilized the membrane of a cell-attached patch instead of rupturing it, thus providing electrical access to the cell without losing the substances necessary for maintenance of ACh sensitivity. We selected nystatin, a pore-forming antibiotic, for this purpose. In initial experiments, nystatin (20–100  $\mu$ M) was added to a patch pipette

containing the normal internal solution (see Methods). Although this method worked and produced much of the results presented in this article, the success rate for obtaining seals was greatly reduced by the nystatin. We then tried to fill most of the pipette with the nystatin-containing solution and to fill the very tip of the pipette with a nystatin-free solution. This improved sealing, but the diffusion of nystatin to the membrane was very unreliable, as the volume of the initially nystatin-free solution was poorly controlled. We finally resorted to obtaining seals in the absence of nystatin and then introducing it (50–100  $\mu\text{g}/\text{ml}$  in internal solution) through a fine polyethylene tube placed inside the patch pipette (Fig. 1 *B*). With the latter method, low series resistance values (4–10  $\text{M}\Omega$ ) were reliably obtained within several minutes after introducing nystatin. The series resistance was then stable or slowly decreased during the course of the experiment (up to 1 h). No suction was applied to the pipette interior once the initial seal was obtained. Nevertheless, an abrupt decrease

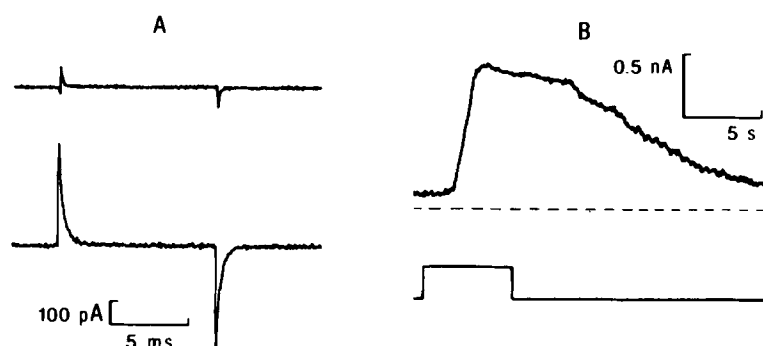


FIGURE 2. ACh-induced current recorded with a nystatin-loaded pipette. (A) Responses to a 4-mV voltage jump from  $-60$  mV with standard cell-attached conditions before (upper trace) and after (lower trace) introduction of nystatin ( $100 \mu\text{g}/\text{ml}$ ) into the recording pipette. (B) The upper trace shows the current response to bath application of  $0.5 \mu\text{M}$  ACh as recorded with the nystatin-loaded pipette. Same cell as in A. Holding potential,  $0$  mV. The lower trace indicates the open/closed status of the valve controlling the microperfusion system (see Methods). The dashed lines indicate the zero-current level.

of the series resistance was occasionally observed, which indicated a spontaneous transition to a classic whole-cell recording mode. When this happened, the cell rapidly developed a large leakage conductance, presumably owing to the entry of nystatin into the cytoplasm, and the experiment was then discontinued. Fig. 2 *A* shows the effect of the addition of nystatin ( $100 \mu\text{g}/\text{ml}$ ) on the capacitive transients measured in response to a 4-mV voltage jump from  $-60$  mV with standard cell-attached conditions. The small capacitance current recorded in the upper trace is due to the pipette and holder input capacitance. Introduction of nystatin is accompanied by the development of an additional capacitive current (lower trace) over a time period of 10 min. This extra capacitance,  $26$  pF in this example, presumably reflects the capacitance of the cell, since it approximately corresponded to the expected capacitance for such cells in standard whole-cell recordings. The access resistance typically decreased from an unmeasurably high level to a value similar to that found

in whole-cell recording. In the experiment of Fig. 2, the access resistance decreased to  $\sim 10 \text{ M}\Omega$ .

The increased capacitance shown in the lower trace of Fig. 2 A suggests that the experimental conditions are electrically similar to those seen in the usual whole-cell recording configuration. In accordance with this idea, we were able to record ACh-induced currents after the capacitance increase was completed. Fig. 2 B shows an example of a Ca-activated K current elicited from the same cell as in Fig. 2 A in

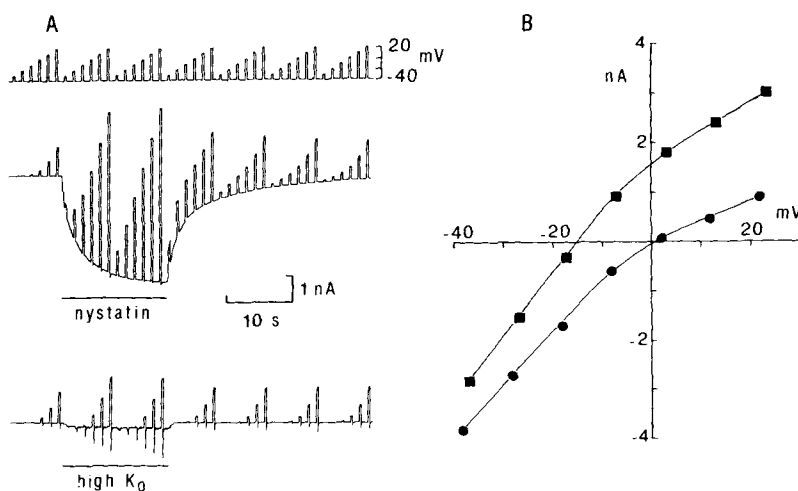


FIGURE 3. Conductance changes associated with application of nystatin to the bath. (A) A cell was subjected to standard whole-cell recording with the usual (nystatin-free) intracellular solution (see Methods). The voltage pulse protocol (top trace) explored the cell conductance between  $-40$  and  $+20$  mV. Upon application of nystatin-free intracellular solution to the bath (labeled "high  $K_o$ "; bottom trace), a small conductance change was observed, owing to the effects of external K ions on the Ca-activated K channel permeability. In the trace shown, 2% methanol was included in the test solution as a check for solvent effects during nystatin application; the methanol-free high- $K_o$  solution gave very similar effects (data not shown). When nystatin ( $100 \mu\text{g/ml}$ ) was applied together with the high- $K_o$  solution, a large conductance change was observed (middle trace). This was totally reversible after an  $\sim 1$ -min wash (not shown). (B)  $I$ - $V$  plot of the nystatin-induced current (circles) obtained by subtracting the  $I$ - $V$  curve without nystatin from that with nystatin in A. The reversal potential was approximately  $+1$  mV. An  $I$ - $V$  curve obtained using a similar procedure (squares), but using the standard intracellular solution diluted by a factor of 2, gave a reversal potential of  $-15$  mV. Various liquid junction potentials have been measured independently and corrected for when plotting the  $I$ - $V$  curves.

response to a brief exposure to  $0.5 \mu\text{M}$  ACh in the bath. In this experiment, the holding potential was  $0$  mV, the approximate reversal potential for Cl (see Methods). In other cells, we recorded Ca-activated Cl currents at  $-80$  mV, near the reversal potential for K, in response to ACh application (data not shown). Both types of ACh-activated current were indistinguishable from those recorded during the first few minutes of whole-cell recording (e.g., see Marty et al., 1984).

In order to characterize further the conductance induced in the membrane by

nystatin, we introduced it into the bath during standard whole-cell recording, as shown in Fig. 3. In this experiment, the pipette contained the usual nystatin-free internal solution, and nystatin (100  $\mu\text{g}/\text{ml}$  in internal solution) was briefly applied to the bath via the rapid-perfusion system described in the Methods. The holding potential was  $-40$  mV and the conductance of the cell was examined during a series of voltage pulses up to  $+20$  mV (top trace in Fig. 3 A). The high- $\text{K}_o$  solution containing nystatin caused a rapid and reversible increase of conductance, as shown in the middle trace of Fig. 3 A. Complete reversibility was seen after  $\sim 1$  min. The

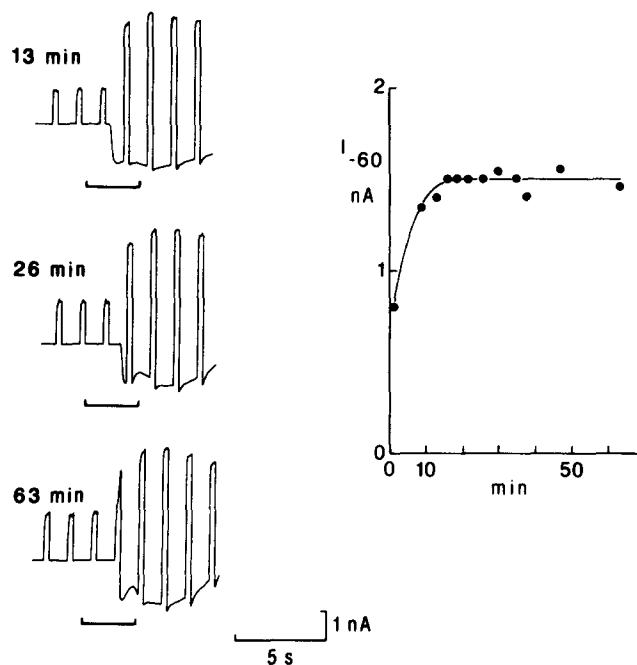


FIGURE 4. Lack of wash-out in recording with the nystatin method. The pipette solution contained 100  $\mu\text{g}/\text{ml}$  nystatin. Cell capacitance, 23 pF; series resistance, 7.1–11.1 M $\Omega$ ; holding potential,  $-60$  mV; test pulses to  $+20$  mV. (Left) Responses to bath applications of 0.5  $\mu\text{M}$  ACh (bars) as recorded at various times after nystatin induced an access conductance in this cell-attached patch. (Right) Amplitude of the ACh-induced current measured at  $-60$  mV as a function of time. For each stimulation, the maximum for current values measured before voltage pulses to  $+20$  mV is plotted. No decline was observed over a 60-min period of recording.

application of the same high- $\text{K}_o$  solution without nystatin had only a small effect on the cell's conductance (bottom trace in Fig. 3 A). Fig. 3 B shows the steady state current-voltage ( $I$ - $V$ ) relationship of the nystatin-induced current (circles). It had a mild inward rectification and reversed at approximately  $+1$  mV, as expected, since the nystatin-induced conductance in bilayers has little selectivity among monovalent cations (Kleinberg and Finkelstein, 1984). An  $I$ - $V$  curve obtained using a similar procedure (squares), but subjecting the cell to solutions diluted by a factor of 2, gave a negative reversal potential ( $-15$  mV). This experiment shows that the concentration

of nystatin used in the experiment of Fig. 2 is able to elicit a large, cation-selective conductance if applied over the entire cell surface. The cationic selectivity is roughly as expected for the one-sided effect of nystatin ( $P_{\text{anion}}/P_{\text{cation}} = 0.1$ ; Marty and Finkelstein, 1975). It should be noted that the response had not reached saturation when nystatin was removed in the experiment of Fig. 3. Longer applications led to uncontrolled, poorly reversible responses. This is to be expected since sustained application to the membrane patch in the cell-attached mode elicits a conductance ( $\sim 10^{-7} \Omega^{-1}$ ) of the same order as that obtained in Fig. 2 by applying briefly the same concentration of nystatin over the entire cell surface.

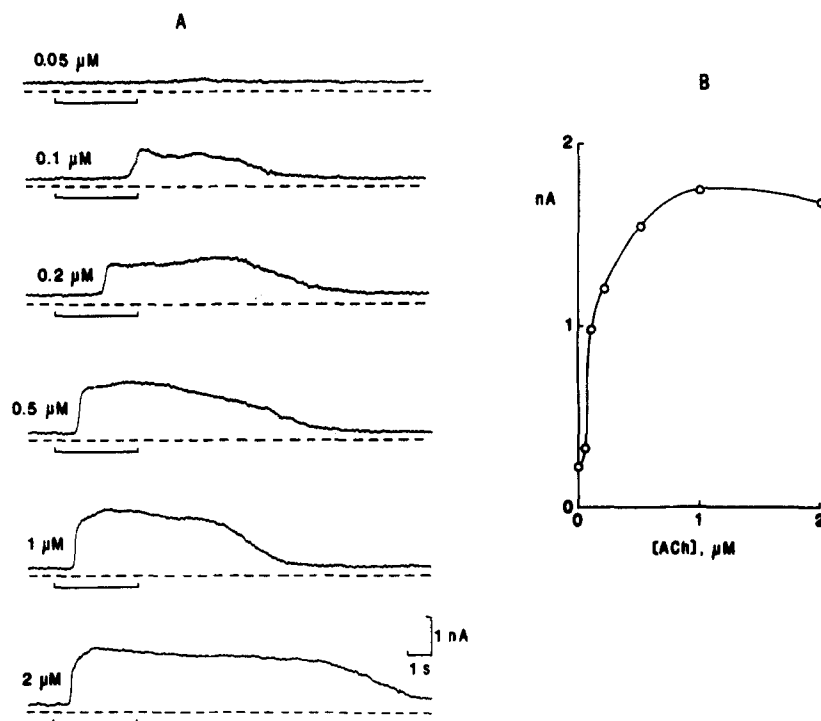


FIGURE 5. Dose-response curve of ACh-induced currents. Currents were recorded at 0 mV using a nystatin-loaded pipette. (A) Responses to ACh applications (brackets below records) in concentrations ranging from 0.05 to 2.0 μM. The dashed lines indicate the zero-current level. (B) Dose-response curve obtained by plotting the maximum current in A as a function of the ACh concentration.

The utility of the nystatin method is shown in Fig. 4. Three responses to the application of ACh (0.5 μM) are shown at 13, 26, and 63 min after the beginning of the experiment. Voltage pulses to +20 mV were given from a holding potential of -60 mV. The ACh-induced current at -60 mV is primarily carried by  $\text{Cl}^-$ , while that at +20 mV is carried by both  $\text{K}^+$  and  $\text{Cl}^-$  (Marty et al., 1984). The peak current at -60 mV showed no decline during the entire recording (Fig. 4). The delay between ACh application and the onset of the current also showed no evidence of wash-out.

*ACh Dose-Response Relationship*

Armed with the nystatin method, we were able to obtain dose-response relationships between ACh and the currents it elicited in these cells. We show here the dose-response curves for Ca-activated K currents at 0 mV. Fig. 5 A shows the responses to six different concentrations of ACh, from 0.05 to 2.0  $\mu\text{M}$ . Note the progressive

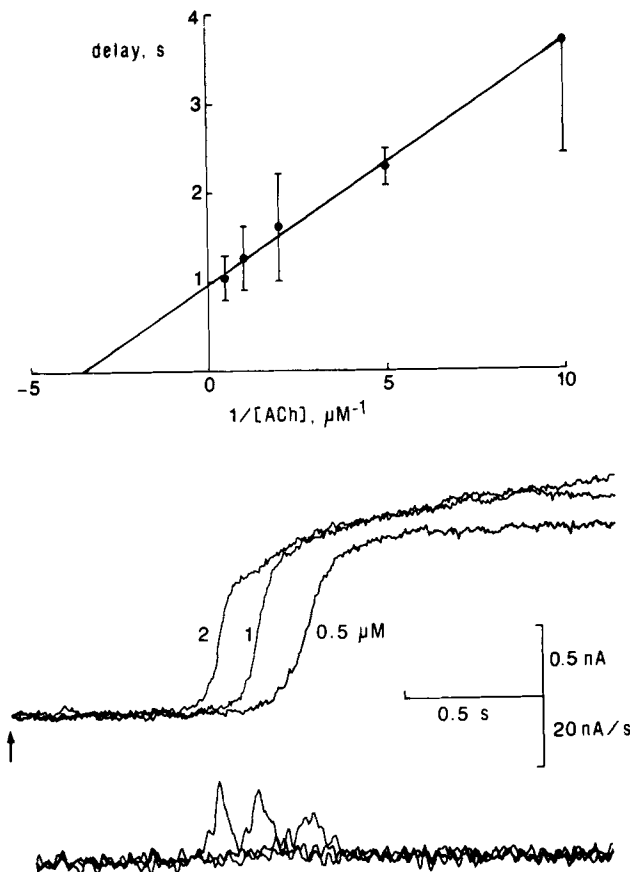


FIGURE 6. Effect of ACh concentration on the response delay. (*Top*) Averages of the delays obtained as illustrated in the lower panel in five different cells. Error bars indicate standard deviations. A linear regression line gives a minimum delay of 0.97 s and an apparent  $K_d$  of 0.27  $\mu\text{M}$ . (*Bottom*) Superimposed traces of the current responses to 0.5, 1.0, and 2.0  $\mu\text{M}$  ACh in the experiment of Fig. 4. The onset of ACh application is indicated by the arrow (beginning of the traces). The lower traces show time derivatives of the current traces.

decrease of the delay and the increasing duration of the response as the agonist concentration was increased. Fig. 5 B plots the peak current as a function of [ACh]. The zero concentration point is the mean of the currents recorded before ACh application for the various trials. The peak current saturated above  $\sim 1.0 \mu\text{M}$ , although the duration of the response increased (see Fig. 5 A). The increasing dura-

tion may have been caused by the time needed to wash away all of the ACh from the cell surface (see Methods). The low value at  $0.05 \mu\text{M}$  ACh gave a sigmoid rise to the dose-response curve.

The delay between ACh application and the onset of the response also approached a minimum value with increasing [ACh]. The delay was measured by digitally differentiating the current trace and selecting the maximum of the derivative, as shown in Fig. 6 (bottom). The three traces are aligned at the time of ACh application, shown by an arrow. The delay decreased monotonically as [ACh] increased. In our experiments, the delay reached a minimum value of 0.7–1.0 s at the highest [ACh], with some variability among preparations. The relationship between the mean delay and  $[\text{ACh}]^{-1}$  for five cells is shown in Fig. 6 (top) and is approximately linear. The minimum delay is estimated by the *Y*-intercept of the regression line and was 0.97 s. The  $K_d$  of ACh binding is estimated by the negative inverse of the *X*-intercept (see model in Discussion), and was  $0.27 \mu\text{M}$  in these experiments.

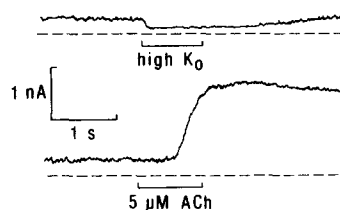


FIGURE 7. Comparison between the delay caused by perfusion and that caused by a high ACh concentration. The pipette solution contained  $100 \mu\text{g/ml}$  nystatin; holding potential, 0 mV. Both traces were obtained with the cell situated in the same position with respect to the microperfusion outlet. The upper trace shows the current response to application of a K-rich (110 mM) solution (delay,  $\sim 30$  ms). The lower trace shows the response to  $5.0 \mu\text{M}$  ACh (delay,  $\sim 0.8$  s).

It is important to ask whether the minimum delay could be caused by an experimental artifact due to the time required to apply ACh. Fig. 7 shows that the response of a cell to a high- $K_0$  solution was very rapid, on the order of  $\sim 30$  ms, by comparison with the  $\sim 0.8$ -s delay seen in response to  $5 \mu\text{M}$  ACh. In the same cell, the delay caused by  $10 \mu\text{M}$  ACh was also  $\sim 0.8$  s (data not shown).

#### DISCUSSION

We have presented a method for prevention of wash-out of ACh sensitivity in rat lacrimal gland cells. The method is based on perforating a cell-attached patch with a multitude of tiny pores formed by nystatin. It is for this reason that we have called the method "perforated patch recording" (Horn et al., 1988). The nystatin channels increase the permeability of the patch to small ions, thus providing electrical continuity between the cytoplasm and the pipette, without the loss of larger substances necessary for the muscarinic response to ACh.

In the following sections, the use of nystatin for this purpose is discussed in terms of the known properties of this substance. We then turn to a discussion of the results on the kinetics of the muscarinic response.

#### *Use of Nystatin to Prevent Wash-Out*

The choice of nystatin in the present experiments was guided by previous work using this compound to permeabilize cells, notably erythrocytes (Cass and Dalmark, 1973) and molluscan neurons (Russell et al., 1977; Tillotson and Horn, 1978). When applied to artificial bilayers, nystatin forms cation- or anion-selective channels, depending on whether it is applied from only one aqueous compartment ("one-sided effect") or from both ("two-sided effect"; Marty and Finkelstein, 1975). These results are in agreement with the present finding of a cation-selective conductance when applying nystatin to the bath solution. The difference between one-sided and two-sided effects relies on the inability of nystatin to cross the bilayer and is by itself a good indication that nystatin will not enter the cell. In our nystatin experiments, there was no visual sign of cell degradation over recording times as long as 1 h, and the cell resistance (measured near the resting potential) was remarkably stable.

Channel openings were not detected during the application of nystatin in the patch pipette. This is not surprising. Given the results obtained in lipid bilayer membranes at high salt concentrations (Kleinberg and Finkelstein, 1984), it is possible to estimate a unitary conductance of  $\sim 0.4$  pS under our experimental conditions. The large conductance induced by nystatin in the patch is therefore a consequence of the large number ( $>10^5$ ) of channels.

Nystatin forms channels with a diameter of  $\sim 0.8$  nm, with a molecular cut-off for sugar molecules at  $\sim 200$  (Holz and Finkelstein, 1970). This sets a lower limit of 200 for the substance(s) responsible for wash-out, in agreement with rough molecular-weight estimates obtained from the analysis of the wash-out time course (Zimmerberg, J., and A. Marty, manuscript in preparation). Alternatively, however, the channels could stop the "wash-out molecules" on the basis of electric charge. Thus, nystatin channels are impermeant both to divalent cations and to divalent anions (Cass et al., 1970; Kleinberg and Finkelstein, 1984).

Altogether, nystatin has a number of properties that make it an attractive choice to obtain a whole-cell recording free from wash-out. First, it is possible to obtain a low and stable series resistance between the pipette and cell (as low as  $2.5$  M $\Omega$ ). Second, nystatin does not enter the cell and thus does not disturb the system under study. Third, it operates a sharp cut-off at a relatively low molecular weight, leaving most cell metabolites unperturbed. Fourth, nystatin channels are not voltage dependent; use of voltage-dependent channels for the same purpose could lead to uncontrolled variations of the access conductance to the cell when giving voltage jumps. Fifth, the method is likely to work on a wide variety of cell types, including red blood cells (Cass and Dalmark, 1973), invertebrate neurons (Russell et al., 1977; Tillotson and Horn, 1978), and tissue-cultured mammalian cells (Horn et al., 1988), because nystatin inserts into the membrane bilayer and does not require cell-specific receptors. The obvious price to pay for the absence of wash-out is the lack of diffusion of high-molecular-weight compounds from the pipette to the cell interior. In the pres-

ent experiments, no effort was made to test whether HEPES and EGTA were in fact able to enter the cell and to buffer the cytosolic proton and Ca levels; however, the selectivity of nystatin suggests that the only substances exchanged between the pipette and cytoplasm were small monovalent ions (primarily  $K^+$ ,  $Na^+$ , and  $Cl^-$ ).

#### *Interpretation of Kinetic Results*

Two aspects of the current kinetics have been demonstrated in the present experiments: (a) the presence of a prolonged delay without any appreciable current rise followed by a rapid increase to the maximum level, and (b) a hyperbolic dependence of the delay on ACh concentration with a minimum value near 1 s for large agonist doses. It should be noted that our kinetic experiments were carried out at a holding potential of 0 mV. Because of the voltage dependence of Ca-activated K channels (Barrett et al., 1982; Findlay, 1984), the kinetics and extent of activation of these channels at more negative resting potentials is likely to be different.

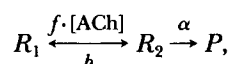
The control experiment shown in Fig. 7 excludes the possibility that the delay could be due to diffusion of ACh to the cell. Another possible rate-limiting step could be the response time of the channels to a rise of Ca. One way to test this response time in principle is to trigger Ca entry through voltage-dependent Ca channels and follow the time course of the ensuing rise of Ca-dependent K current. This is not possible in exocrine glands, where voltage-dependent Ca channels are lacking. Chromaffin cells, however, possess both voltage-dependent Ca channels and Ca-activated K channels. After depolarization, Ca entry induces an increase in Ca-activated K current that is detectable within 2 ms and reaches its maximum within 40 ms (Marty and Neher, 1985). Thus, the delay is two to three orders of magnitude too large to be attributed to kinetic properties of Ca-activated K channels. In the following, we assume that the time course of Ca-activated K current faithfully reflects the underlying changes in  $[Ca]_i$ .

The abrupt increase of K current after a long delay suggests that  $[Ca]_i$  increases suddenly, in a regenerative fashion, after the delay. The control of  $[Ca]_i$  therefore depends on a positive feedback system. The presence of such a system was in fact previously inferred from observations on the response to a Ca ionophore, to ACh, and to GTP $\gamma$ S (Marty et al., 1984; Evans and Marty, 1986). The molecular mechanism involved in this regulation remains to be elucidated.

Because of this positive feedback, the notion of a dose-response curve takes on a significance that is very different from that pertaining to neuromediator-induced current responses caused by direct receptor-channel coupling. In our experiments, the response amplitude is constant at  $[ACh] > 0.5 \mu M$ , and the agonist concentration only influences the delay of the response. This suggests that the Ca response has a stereotyped shape and that the agonist concentration mainly determines the time at which this Ca transient occurs. An alternative explanation would be that the Ca response is in fact graded with the agonist dose, while the current dose-response curve takes its sigmoid shape as a result of a nonlinear dependence of Ca-activated K current activation on  $[Ca]_i$ . Although saturation of such currents does occur at high  $[Ca]_i$  (Trautmann and Marty, 1984), explaining the large variations of the delay with  $[ACh]$  on the basis of this graded Ca hypothesis would require in addition that,

for small variations of  $[Ca]_i$ , the probability of opening of Ca-activated K channels does not change. There is no sign, however, of such behavior in the dose-response curves obtained with these channels as a function of  $[Ca]_i$  (e.g. Barrett et al., 1982; Findlay, 1984).

One simple way to explain the dependence of the delay on  $[ACh]$  is as follows. We assume that the delay is determined by the time needed to obtain a threshold concentration  $[P]_0$  of a certain product  $P$ . We further assume that the rate of formation of  $P$  is proportional to the number of receptors that have bound ACh. Thus,



where the binding of ACh is represented by the first step and the second step is rate-limiting with a rate  $\alpha$ . An apparent activation rate can be expressed as  $\alpha'$ , where

$$\alpha' = \alpha \cdot [R_2],$$

and

$$[R_2] = [R_T] \cdot f \cdot [ACh] / (f \cdot [ACh] + b),$$

$[R_2]$  being the concentration of bound receptors, and  $[R_T]$  being the total concentration of receptors. The delay  $d$ ,  $[P]_0$ , and  $\alpha'$  are related by

$$[P]_0 = d \cdot \alpha'.$$

Hence,

$$d = ([P]_0 / \alpha \cdot [R_T]) \cdot \{1 + (b/f) \cdot [ACh]^{-1}\}.$$

For this model, the delay is a linear function of  $[ACh]^{-1}$  with a minimum value of  $[P]_0 / \alpha \cdot [R_T]$  at high  $[ACh]$ . The negative inverse of the X-intercept in this model is  $b/f$ , which is the  $K_d$  for ACh binding. Fig. 6 (top) shows that such a model is a good description of our data. One possible interpretation of this scheme is that  $P$  represents the active form of phospholipase C, the enzyme responsible for the production of  $IP_3$ , while  $\alpha$  would be a lumped rate constant representing various events taking place between receptor occupancy and activation of phospholipase C. According to present views, these events involve the activation of a GTP-binding protein and diffusion steps representing the sequential encounter of the receptor, the GTP-binding protein, and phospholipase C (Evans and Marty, 1986). Alternatively, the compound  $P$  may represent some other substance in this pathway.

An interesting feature of this model is that it assumes a 1:1 stoichiometry for the binding of ACh to its receptor. This in no way contradicts to the finding of a sigmoid dose-response curve (Fig. 5), since this feature is attributed to regenerative feedback taking place downstream from ACh binding.

We thank the Ecole Normale Supérieure and the Université Pierre et Marie Curie for the financial support of R. Horn during his sojourn in France.

*Original version received 29 October 1987 and accepted version received 22 January 1988.*

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