Muscarine and t-LHRH Suppress M-Current by Activating an IAP-Insensitive G-Protein

Paul Pfaffinger

Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98195

The control of M-current by muscarinic ACh receptors and luteinizing hormone releasing hormone (LHRH) receptors was studied in dialyzed frog sympathetic ganglion neurons. M-current was recorded in dialyzed cells without run-down or changes in its biophysical properties and could be reversibly suppressed by muscarine and teleost LHRH (t-LHRH). However, dialysis with internal solutions lacking ATP or substituting with APP(NH)P caused the loss of M-current, suggesting that dephosphorylation suppresses the activity of M-channels. M-current over-recovers after agonist addition and removal to a size 30% larger than control, as if latent channels are activated during the recovery. Dialysis of cells with the G-protein activators GTP γ S, fluoride, and aluminum fluoride causes loss of M-current. G-protein activation by receptors was confirmed by dialysis with low concentrations of GTP γ S in competition with GTP. This prevents the rapid loss of M-current, but addition of muscarine or t-LHRH caused irreversible loss of M-current, suggesting that both transmitter receptors do suppress M-current by activating a G-protein. Suppression of M-current was not affected by treatment with 0.1 μ g/ml pertussis toxin (IAP) for 24–48 hr. In addition, based on the lack of IAP-specific labeling of frog sympathetic neuron membrane proteins, no IAP-sensitive G-proteins are present in these cells. These results indicate that an IAP-insensitive G-protein couples muscarinic and LHRH receptors to the suppression of M-current.

Agonist receptors modulate the excitability of frog sympathetic neurons by regulating a potassium current called the M-current. This current is suppressed when ACh acts on muscarinic receptors, generating a slow EPSP (Brown and Adams, 1980). It is also suppressed by a peptide immunogenically similar to teleost luteinizing hormone releasing hormone (t-LHRH), generating a late, slow EPSP (Eiden et al., 1982; Jan et al., 1983; Jones et al., 1984). M-current is also suppressed by other small-

molecule "transmitters" (Adams et al., 1982b, 1983). Because several receptors can suppress the M-current, they must converge at some level along a common pathway. However, the biochemical pathway controlling M-current in sympathetic neurons is not known; thus, little is understood about the mechanism of convergence.

M-current-like conductances have been recorded in other neurons, including hippocampal pyramidal neurons (Halliwell and Adams, 1982) and differentiated NG108-15 cells (Higashida and Brown, 1986), as well as in smooth muscle (Sims et al., 1985). In NG108-15 cells, the suppression of M-like current by bradykinin is reported to involve the activation of pertussis toxin (IAP)-sensitive GTP-binding proteins (Higashida et al., 1986). These IAP-sensitive G-proteins activate phospholipase C, and the suppression of M-current has been attributed to phosphorylation by protein kinase C (Higashida and Brown, 1986). On the other hand, protein kinase C is not thought to be involved in the control of the M-like current in hippocampal neurons (Malenka et al., 1986). Therefore, the mechanism controlling M-current seems to be different in different cells.

Adams et al. (1982b) explored the intracellular control of M-current by introducing molecules into bullfrog sympathetic neurons ionophoretically. The M-current was unaffected in that study by ionophoresis of cAMP, cGMP, guanosine-5'-O-(3-thiotriphosphate (GTP γ S), Ca²⁺, or EGTA. However, ionophoresis suffers from the limitation that only a small and often unknown amount of the substance is injected into these large cells (> 50 μ m). In principle, better control could be obtained by using whole-cell dialysis with gigaseal pipettes. My work shows that conditions can be found that preserve a stable M-current in dialyzed cells. Using this technique to change intracellular nucleotides and small ions, I could examine whether GTP-binding proteins (G-proteins) couple muscarinic ACh and/or LHRH receptors to the suppression of M-current in frog sympathetic neurons.

Materials and Methods

Preparation of isolated sympathetic neurons. The abdominal chains of the sympathetic nervous system of 6–10 cm frogs (*Rana pipiens*) were removed and placed in frog Ringer's for 15–30 min at 5°C. The chains were desheathed and the individual ganglia cut out, the larger ones being cut into pieces for better enzyme access. These pieces were treated with 0.1% collagenase (Sigma, Type I) + 0.05% trypsin (Sigma, bovine) in 2 ml frog Ringer's for 1.5–2.0 hr at 26–28°C. The partially dissociated ganglia were then teased apart with glass needles, and the cell-enzyme mixture was triturated 3 times and placed into culture medium at a ratio of 1:3 at 5°C until used. The culture medium was 75% Leibovitz's L-15 medium (Gibco), 20% distilled water, and 5% fetal calf serum (Gibco). This procedure yields cells that remained viable for up to 3 d.

Recording solutions. The intracellular solutions are listed in Table 1. The protease inhibitor leupeptin enhanced the size and the stability of

Received Aug. 27, 1987; revised Feb. 11, 1988; accepted Feb. 12, 1988.

This work was supported by NIH Grant NS08174 to Dr. Bertil Hille, NIH Grant HL30639 to Dr. Neil Nathanson, NIH Training Grant GM07270, and an ARCS Foundation fellowship to Paul Pfaffinger. I wish to thank Drs. Bertil Hille and Neil Nathanson for their continued support and assistance, Drs. Charles Luetje and Jennifer Martin for their help with IAP experiments, Lea Miller for help typing this manuscript, Don Anderson for his technical assistance, Don McBride for his programs and programming assistance, and Drs. Mark Leibowitz, Steven Barnes, and Martha Bosma for their many helpful discussions and assistance in preparing the manuscript.

Correspondence should be addressed to Paul J. Pfaffinger, Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 722 W. 168th Street, New York, NY 10032.

Copyright © 1988 Society for Neuroscience 0270-6474/88/093343-11\$02.00/0

Table 1. Composition of internal solutions

Constituent ^a	Normal	Fluoride	0 ATP/ 0 Mg ²⁺	0 ATP	APP (NH)P
Na ₄ ATP	1.5	1.5	—	—	—
Li ₄ APP (NH)P	—	—	—	—	1.5
K Aspartate	100	100	100	100	100
KCl	20	20	20	20	20
NaCl	5	5	5	5	5
MgCl ₂	1–2	1	—	1	1
EGTA	1	1	—	1	1
EDTA	—	—	5	—	—
GTP (μM)	(100)	100	—	100	100
Leupeptin (μM)	100	100	100	100	100
KF (NaF)	—	10	—	—	—
AlCl ₃ (μM)	—	(100)	—	—	—
MOPS	5	5	5	5	5
pH	7.4	7.4	7.4	7.4	7.4

^a Concentration in mM except as noted.

muscarinic responses. Fresh leupeptin stock was made up from powder each week and added to the internal solution on the day of the experiment. In experiments using fluoride, 10 mM NaF or KF, either with or without 100 μM AlCl₃, was added to the standard intracellular solution. For experiments using guanine nucleotide derivatives, 100 μM GTP was omitted from the standard intracellular solution, unless stated otherwise. A frozen stock of the desired derivative was thawed and added to the internal solution on the day of the experiment. Since many nucleotide derivatives come as Li salts, control experiments were run with an equal concentration of Li added to the standard internal solution. In these control experiments, Li had no effect on M-current or its control by agonists.

The standard bath solution was 2.5 mM K Ringer's: 115 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 4 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.3. Any drugs were added to Ringer's solutions before the experiments and were then applied by exchanging the bath solution. Atropine and muscarine were made up as 2 mM stocks in 2.5 mM K Ringer's. t-LHRH was aliquoted into 100 mM acetic acid, lyophilized, and stored frozen. One aliquot would be made into a stock and kept frozen for up to 2 weeks. Fresh t-LHRH containing Ringer's was made up each day.

Electrophysiological recordings. Cells were voltage-clamped with the whole-cell voltage clamp of Matteson and Armstrong (1986). Pipettes had tip resistances of 0.6–1.0 MΩ, with openings of 3–8 μm. Only cells that formed gaseals were used. The currents were low-pass filtered at 500 Hz, digitized, and recorded on an IBM PC-XT computer using the BASIC FASTLAB software and hardware package (INDEC Systems, Sunnyvale, CA). Analysis was done using programs of the BASIC FASTLAB system.

Absolute-zero current was determined by recording the current signal with the pipette tip in air, either before or after the actual experiment, and is shown in all figures by a dashed line. Membrane currents were recorded without leak subtraction. All cells had some linear background currents that needed to be subtracted later to determine the true size of the M-current. The linear current, measured at potentials more negative than –75 mV, was subtracted from the currents measured at potentials more positive than –75 mV to determine the true size of the M-current. Recorded voltages were corrected for a junction potential by subtracting the measured 10 mV junction potential.

The recording chamber was created by sealing a plastic barrier to a 35 mm tissue culture with petroleum jelly. The barrier formed 3 connecting wells. Cells in tissue culture medium were pipetted into the large center well immediately before the recording. The 2 side chambers were for solution inflow and outflow and the bath electrode, an agar bridge connected to a silver–silver chloride pellet with 150 mM NaCl. After being pipetted into the chamber, cells were allowed to settle for several minutes, and then the tissue culture medium was exchanged for normal frog Ringer's. The solution change was gentle enough to ensure that most cells remained in the recording chamber. A suitable isolated cell was then chosen and a seal attempted. Once the gaseal formed, the

cell was lifted off the bottom of the dish by backing-up the pipette. Subsequent solution changes could then be made much more rapidly; however, the dead time for solution exchange was still 10–15 sec. Six times the total bath volume was delivered within 60 sec. These techniques produced recordings lasting over 1 hr on cells with very low linear leak conductances over many solution changes. A new tissue culture dish with fresh cells was used for each recording.

Pertussis toxin (IAP) experiments. Before being treated with IAP, the cells were first allowed to precipitate for at least 6 hr in a tube containing culture medium plus 1% penicillin–streptomycin at 5°C. This culture medium was carefully drawn off and exchanged for new medium lacking antibiotics. To test for block of function with IAP treatment, cells were placed in fresh culture medium, either with or without 0.1 μg/ml IAP, and kept at 25°C for 24–48 hr. Electrophysiological recordings were performed as described above. The only noticeable difference between these cells and ones held at 5°C was a tendency to aggregate at the higher temperature.

Other experiments sought to identify IAP-sensitive G-proteins in gels. Sympathetic neurons were prepared identically to those used in recordings. After 24 hr at 25°C in a medium either with or without IAP, cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, and 0.9 mM CaCl₂, pH 7.4) and then washed and homogenized in 100 mM Tris, pH 8.0. The membranes were washed twice with Tris and then kept on ice until used for labeling. Brain membranes were prepared by homogenization of whole brains in 100 mM Tris, pH 8.0, followed by 2 washes with Tris. If membranes were not used immediately, they were frozen in a dry ice/ethanol bath and kept at –70°C until used. Labeling with ³²P-nicotinamide-adenine dinucleotide (³²P-NAD) and IAP was done in Dr. Neil Nathanson's laboratory (Dept. of Pharmacology, University of Washington) with the help of Drs. Charles Luetje and Jennifer Martin. Labelings were attempted with membranes and with solubilized membrane proteins (Halvorsen and Nathanson, 1984; Pobiner et al., 1985). After labeling, the proteins were separated on a 1-dimensional SDS-electrophoresis gel and analyzed by autoradiography. Sympathetic neurons from 6–10 frogs were used per lane; based on Lowry assays, there are 20–25 μg of sympathetic ganglion membrane protein per frog.

Materials. *Rana pipiens* frogs were obtained from Kons Biological Supplies; Na₄GTP, 5'-adenylylimidodiphosphate [Na₄APP(NH)P], 5'-guanylylimidodiphosphate [Na₄GPP(NH)P] from P-L Biochemicals; guanosine-5'-O-(3-thiotriphosphate) (Li₄GTPγS) and guanosine-5'-O-(2-thiodiphosphate) (Li₄GDPβS) from Boehringer Mannheim Biochemicals; t-LHRH from Peninsula Biochemicals; Na₃ATP, muscarine, atropine, leupeptin, collagenase Type I, and trypsin from Sigma Chemical Company; Leibovitz's L-15, 10,000 unit penicillin–streptomycin, and fetal calf serum from Gibco; and ³²P-NAD was either purchased from New England Nuclear or prepared by Dr. Charles Luetje; IAP was prepared by Dr. Jennifer Martin.

Results

The long-term goal of this study is to gain a better understanding of the coupling between receptor occupancy and the suppression of M-current. I first evaluated the quality of dissociated sympathetic neurons and the ability to record M-current in dialyzed isolated cells.

The cells used had somas of 30–50 μm along the major axis and lacked any processes. Before recording the only obvious intracellular particle was the nucleolus. Cells with an axon were not used because of the difficulty in dialysis and voltage-clamping of the axon, and any cell showing obvious damage from the dissociation such as blebbing was rejected.

In good recordings the seal and breakthrough occurred without forming a bleb across the pipette tip. Since the pipette openings used were quite large (3–8 μm diameter), bleb formation was a problem whenever the gaseal formed slowly, resulting in electrical access to the cell interior without good dialysis of the cell (Lindau and Fernandez, 1986). Occasionally, the bleb broke spontaneously, yielding good access to the cell. The quality of dialysis was evaluated visually and electrically. In a poorly dialyzed cell there is no change in the appearance of the cell

Figure 1. Response of a dialyzed sympathetic ganglion neuron to muscarine. Normal internal solution. *A*, Gating of M-current as the membrane potential was stepped from -35 to -60 mV for 200 msec. *B*, Hyperpolarizing test pulses are repeated every 5 sec as Ringer's containing $20 \mu\text{M}$ muscarine was added to the bath, resulting in a 60% decrease in the size of the M-current. *C*, Current recorded 25 sec after beginning the solution change. *D*, About 5% of the M-current reappeared in the 35 sec between *C* and *D*, reflecting the desensitization that occurs in dialyzed cells. (The voltage pulse in *D* lasted 400 msec.) Dashed line indicates zero current.

even after 15–30 min of recording. By contrast, in a well-dialyzed cell the entire nucleus becomes easily visible with Hoffmann optics and the cytosol less dense in appearance. Usually, tiny particles can be seen inside the tip of the recording pipette, probably indicating larger structures that have diffused out of the cell. Electrically, good access corresponded to low access resistances (generally $<2.5 \text{ M}\Omega$) and good voltage-clamp control of the large ($>2 \text{ nA}$) and fast, voltage-gated sodium current. Only well-dialyzed cells were used in these experiments, unless otherwise stated.

Besides sodium current, dialyzed cells contained Ca currents, and several types of potassium currents including A-current, delayed rectifier current, and M-current. Ca-activated currents were poorly resolved because of Ca buffering by the intracellular solution. The potassium currents tend to be smaller than those reported from *Rana catesbeiana* neurons but were otherwise identical.

The M-current

The noninactivating M-current was isolated from other inactivating potassium currents by holding the cell at -35 mV (Fig. 1*A*). When the membrane potential is stepped to -60 mV, the standing current of 0.2 – 2.0 nA undergoes both an instantaneous and a time-dependent decrease. The instantaneous decrease occurs because of the decreased electrical driving force on K ions. The time-dependent decrease is characteristic of the voltage-gated closing of M-current. As seen in Figure 1*A*, the instantaneous current step upon depolarizing the cell to -35 mV is much smaller than the jump when stepping to -60 mV, indicating that many channels are gated closed at -60 mV. The remaining current reappears with a time constant characteristic of the voltage-gated activation of M-current.

As reported by Brown and Adams (1980), the M-current is suppressed by muscarine. Figure 1*B* shows the response of a dialyzed cell to perfusion of $20 \mu\text{M}$ muscarine. The cell was dialyzed for 4 min at -35 mV. M-current amplitude was monitored by a step to -60 mV every 5 sec. As can be seen, the standing current at -35 mV declines following the application of $20 \mu\text{M}$ muscarine to the bath. In addition, the steps to -60 mV produce smaller instantaneous and time-dependent decreases in current (Fig. 1*C*). The slow response to muscarine here is characteristic of the time taken to exchange the bath and is not a measure of the intrinsic speed of the response. There is a slight desensitization of M-current suppression, with about 5–10% of the M-current reappearing per minute in the continued presence of $20 \mu\text{M}$ muscarine (Fig. 1*D*). The muscarinic suppres-

sion of M-current is completely prevented by $1 \mu\text{M}$ atropine and is therefore a specific effect of muscarinic ACh receptors.

M-current in isolated dialyzed cells is also suppressed by t-LHRH, a peptide immunogenically identical to the transmitter responsible for the late, slow EPSP (Eiden et al., 1982; Jan et al., 1983). Figure 2 shows the response of a cell as the bath solution is changed to one containing $1 \mu\text{M}$ t-LHRH. The M-current was recorded at -35 mV with 200 msec voltage steps every 5 sec to -60 mV. As with muscarine addition, the current flowing at -35 mV decreases when t-LHRH is added to the bath. The instantaneous step in current as the cell is pulsed to -60 mV becomes much smaller, and the closing and reopening transients are greatly reduced, indicating that very few M-channels remain active. In this experiment, the M-current was reduced by 80%. When the t-LHRH is washed out, the M-current reappears with a time course of 20–30 sec, similar to that seen with muscarine. As in the response to muscarine, there is a slight desensitization of the suppression by t-LHRH, with 7%

Figure 2. Reduction of M-current in a $1 \mu\text{M}$ t-LHRH. *A*, Control trace after 30 min of dialysis using the high- $\text{Na}^+/\text{Mg}^{2+}$ internal solution. Potential steps to -60 mV for 200 msec. *B*, Test pulse was repeated every 5 sec as the bath solution was exchanged for one containing $1 \mu\text{M}$ t-LHRH. *C* and *D*, M-current reappeared in t-LHRH at a rate of 7% of the original M-current per minute, reflecting desensitization. *E*, Pulses every 5 sec as t-LHRH was washed out of the bath with normal Ringer's. *F*, After washing out t-LHRH, the M-current in this cell was 20% larger than before t-LHRH was added.

Figure 3. Properties of the muscarine-sensitive current. *A*, Cell was held at -35 mV and the voltage stepped for 400 msec to potentials between -30 and -100 mV in 10 mV increments. Dashed line is zero current. A reversal potential of -90 mV was measured from the gating transients. *B*, Conductance versus potential for the experiment in *A*. *C*, Time constant for the voltage-dependent closing of the M-current channels. *D*, Loss of M-current in cell dialyzed with reduced internal solution lacking ATP and divalent cations. The M-current was recorded at -30 mV. Breakthrough to whole-cell dialysis condition was at time 0 sec. M-current is maintained in this solution for almost 2 min and suddenly begins to fall. Curve drawn through late time points is an exponential function with 100 sec time constant.

of the M-current reappearing spontaneously per minute during continuous applications of t-LHRH.

Comparison of responses to muscarine and t-LHRH

In these experiments with isolated dialyzed sympathetic neurons, t-LHRH is consistently a more potent agonist than muscarine. Applications of $1 \mu\text{M}$ t-LHRH caused an average reduction in M-current of 88% compared with an average 52% reduction with $100 \mu\text{M}$ muscarine. Over the course of these experiments, about 10% of the cells lacked responses to muscarine altogether. However, when subsequently tested with t-LHRH, these same cells showed normal responses, suggesting they lacked functional muscarinic ACh receptors. The converse situation was more rare: Only 2 cells were recorded that lacked responses to $1 \mu\text{M}$ t-LHRH but responded to muscarine.

In dialyzed cells, the M-current became about 30% larger after muscarine or t-LHRH was washed off than before the drugs were added (see Fig. 7*A*). I have called this "over-recovery." The M-current amplitude generally did not increase beyond this level following multiple drug applications. The current increase was often maintained, although in many cells the M-current returned to its original size if left to recover for several minutes.

Finally, I have noted in dialyzed recordings that a previously undescribed outward rectifying K-current, I_{KP} , is activated by both muscarine and t-LHRH. I_{KP} is inactivated at -35 mV during a normal recording of M-current, but it can be recorded

after removing inactivation by prepulsing the voltage to a potential more negative than -70 mV. I_{KP} inactivation has a time constant of about 5 sec at -35 mV, distinguishing it from the relatively rapid inactivation of A-current. I_{KP} is generally larger in well-dialyzed cells; therefore, it may normally be suppressed by some component that is present in the cytoplasm. Application of muscarine or t-LHRH suppresses M-current, but following prepulses to potentials more negative than -70 mV, more I_{KP} is recorded than before application of agonist. This activation is partially reversible since most of the I_{KP} current is lost following washout of agonist. Because I_{KP} activates in a voltage range similar to M-current, I generally avoided pulse patterns that activate I_{KP} when measuring M-current.

Properties of M-current in dialyzed cells

Since previous investigators failed to record stable M-current using whole-cell voltage clamp, I next examined the properties and stability of M-current in dialyzed cells. I first compared the properties of the muscarine- and t-LHRH-sensitive current in these dialyzed cells to the properties reported for M-current in previous microelectrode studies (Brown and Adams, 1980; Adams et al., 1982a). Figure 3*A* shows a whole-cell recording of M-current in a cell dialyzed with the normal intracellular solution. This cell has a standing outward current of 475 pA at -35 mV. The conductance at the end of a 400 msec voltage step is plotted versus potential in Figures 3*B*. It shows the char-

acteristic steep increase at potentials positive to -60 mV as expected for M-current. The time constant of the voltage-dependent closing of the current decreased from a value of 130 msec at -40 mV to 30 msec at -80 mV (Fig. 3C), numbers very similar to those from microelectrode recordings of M-current. The current is potassium selective based on the reversal potential change with external K concentration from -90 mV in 2.5 mM K to -40 mV in 25 mM K. Finally, the sensitivity of this current to Ba blockade is like that reported in microelectrode studies of M-current, with complete block at 5 mM external Ba. Therefore, this current is the M-current.

I next compared the size and closing rates of M-current immediately after breakthrough with those measured after the cell was well dialyzed. The only consistent effect of dialysis with the standard internal solution was a slight increase in the M-current size during the first 2 min of dialysis, after which the M-current shows little or no change in amplitude [after 360 sec M-current was 1.12 ± 0.08 ($n = 7$) of the initial size]. The closing time constants showed no change over this time. The only significant alteration in M-current occurred late in a recording, when the cell began to deteriorate, as judged visually by the formation of blebs on the surface and electrically by an increase in leak current. When this happened, the M-current generally became smaller and its gating kinetics became faster. It is not clear what triggers this deterioration, although it occurs only after extensive dialysis of the cell.

ATP dependence of M-current

Having confirmed the stability of M-current in my standard internal solution, I examined whether M-current could be recorded using a simpler internal solution containing 5 mM EDTA, no added divalent ions, and no nucleotide triphosphates. Good dialysis with this reduced internal solution was obtained on 5 cells. In all 5 cells the M-current disappeared as the dialysis proceeded, much as has been reported for Ca current in other cells. After 260 sec, M-current was 0.40 ± 0.11 ($n = 5$) of its original size, and after 570 sec, was down to 0.25 ± 0.06 ($n = 4$) and still falling. The loss of M-current with dialysis in this solution typically displayed a threshold phenomenon, where M-current was well maintained for some time until finally the dialysis proceeded to a point where M-current began to fall quickly. Figure 3D shows the loss of M-current in one such cell. M-current was within 90% of its original size for over 100 sec. In the following 100 sec, more than 60% of the M-current is lost. This threshold may represent the time needed to dialyze away the cytosolic nucleotide triphosphate and/or divalent cations sufficiently.

To determine whether this suppression of M-current was due only to a loss of ATP, I dialyzed cells with an internal solution identical to the standard internal solution, only lacking ATP. In well-dialyzed cells the M-current still decayed with this solution. After 225 sec, M-current was 0.55 ± 0.07 ($n = 3$) of its original size, and after 450 sec, 0.19 ± 0.06 ($n = 3$). If anything, the loss of M-current was more rapid, with 1 mM EGTA and 1 mM Mg than if divalents were completely chelated with 5 mM EDTA.

This ATP dependence could imply that the active channel binds ATP or it could indicate that phosphorylation of some essential site is required to maintain functional channels. To test these possibilities, I dialyzed cells with an internal solution identical to the standard internal solution, only substituting the nonhydrolyzable derivative APP(NH)P for ATP. With a K

channel from islet B cells that is shut by ATP, APP(NP)P is able to substitute for ATP (Cook and Hales, 1984). But with the M-channel, APP(NH)P does not substitute. In all 4 sympathetic neurons well dialyzed with APP(NP)P, the M-current was lost, indicating that functional M-channels require hydrolyzable ATP.

In 2 experiments testing the importance of ATP, the access resistance was poor. One had the 0 ATP/1 mM EGTA–1 mM Mg solution and the other, APP(NH)P. In both, the M-current amplitude decreased over time, only more slowly, and some M-current remained even after 10 min. Addition of $1 \mu\text{M}$ t-LHRH to these cells suppressed the remaining M-current as usual, but it did not recover even after extensive washing of the bath. Although too few cells were recorded in this manner to reach any firm conclusion, these results suggest that phosphorylation may be needed in the normal recovery of M-current, and hence that dephosphorylation of this essential site may play a role in the suppression of M-current by agonists.

GTP γ S mimics transmitter responses

I next examined whether GTP-binding proteins (G-proteins) couple muscarinic ACh and/or t-LHRH receptors to the suppression of M-current in frog sympathetic neurons. I first dialyzed cells with a GTP-free internal solution to determine if depletion of cytosolic GTP could eliminate coupling. However, even after dialysis for more than 30 min without added GTP, cells could still respond to muscarine or t-LHRH. This result may reflect the inability of the whole-cell recording technique to dialyze cells of this size (30–50 μm) completely. The high affinity of G-proteins for GTP, binding affinities in the tens of nanomolar range, requires the removal of virtually all the cellular GTP to prevent G-protein activation. In a cell of this size the quality of dialysis may be limited by time requirements or by the actions of cellular enzymatic pathways producing GTP. Therefore, other kinds of experiments were done to test if a G-protein might be involved.

I examined if dialysis with relatively high concentrations of the irreversible G-protein activator GTP γ S in the pipette solution could mimic the agonist suppression of M-current [test (e) of Stryer and Bourne, 1986]. In other systems, GTP γ S at such high concentrations usually activates G-proteins in the absence of agonists. Since M-current is quite stable in cells dialyzed with the standard internal solution, "spontaneous" loss of M-current would implicate a G-protein. Figure 4 (upper left) shows currents in a cell recorded with 50 μM GTP γ S in the internal solution using a large-tipped pipette that dialyzed the cell well. Figure 4 (upper left, A) shows the M-current recorded immediately after breaking into the cell. The cell was held at -35 mV and stepped every 15 sec to -40 mV for 200 msec followed by -80 mV for 200 msec and then depolarized to -35 mV [Fig. 4 (upper left, B)]. The step to -40 mV shows the size of the M-current, while the step to -80 mV was done to show that there is no change in current at a potential where the M-current conductance is already gated closed. The M-current declined steadily after the whole-cell recording was established, while there is little to no change in the current at -80 mV [Fig. 4 (upper left, B–D)]. The loss of M-current was apparently permanent since it did not reappear even after holding for over 10 min ($n = 3$). If the same experiment was done using very small-tipped pipettes that did not dialyze cells well, only 12% of the M-current was lost in 5 min. However, addition of muscarine to such cells caused a complete loss of M-current that was not reversed even after addition of atropine ($n = 3$).

Figure 4. Upper left, Spontaneous loss of M-current with an internal solution containing 50 μM GTP γ S. Time after breakthrough is indicated. The 3 expanded traces shown were all recorded using the voltage step shown under the first trace. *A*, Immediately after breakthrough. *B*, Cell was then held at -35 mV and pulsed every 15 sec (compressed traces). The voltage pulse pattern (200 msec step to -40 mV, then to -80 mV for 200 msec, and finally returned to -35 mV) recorded both the M-current at -35 and -40 mV and the current flowing at -80 mV, where the M-current is gated closed. There is loss of M-current, with little change in the current at -80 mV. Eventually the M-current is completely lost (*C* and *D*). Lower left, Loss of M-current with fluoride-containing internal solution. *A*, Ten seconds after breakthrough. The step to -60 mV lasts 200 msec. *B*, Same voltage pulse repeated every 5 sec. *C*, Last trace is a 400 msec voltage pulse to -60 mV showing that virtually all the M-current has been lost after 220 sec. Right, Comparison of M-current loss with aluminum fluoride-, fluoride-, or GTP γ S-containing internal solutions. The lines are exponentials with the following time constants: 50 μM GTP γ S (triangles), 120 sec; 10 mM NaF (circles), 50 sec; 10 mM NaF + 100 μM AlCl₃ (crosses), 12 sec.

Dialysis with fluoride

A second test for a G-protein is dialysis with an internal solution containing fluoride. Fluoride apparently reacts with trace aluminum to form the aluminum fluoride compound AlF_4^- , which activates GDP-bound G-proteins (Sternweis and Gilman, 1982; Bigay et al., 1985). Cells were dialyzed with internal solutions containing either 10 mM NaF or KF to determine the effect on the M-current. Figure 4 (lower left) shows a cell dialyzed with an internal solution containing 10 mM NaF. The cell was held at -35 mV and pulsed to -60 mV every 5 sec to measure the size of the M-current and its gating transients. Only the pulses are shown. Note the rapid decrease in the size of the M-current in this cell. In 7 out of 7 cells the M-current was rapidly and completely lost after dialysis with F^- -containing internal solutions.

The loss could be accelerated by adding 100 μM AlCl₃ to the internal solution to raise the concentration of AlF_4^- . For all experiments with F^- , the rate of loss of M-current was faster than with internal solutions containing GTP γ S. Figure 4(right) compares the time course of M-current loss in 3 cells, one dialyzed with 50 μM GTP γ S, one with 10 mM NaF, and the last with 10 mM NaF + 100 μM AlCl₃. The loss of M-current in these experiments can be described by exponential functions over the late time points, giving time constants of about 120 sec for GTP γ S, 50 sec for NaF, and 10 sec for NaF + AlCl₃. Fluoride activation may be faster than GTP γ S because it activates G-proteins in the GDP-bound state, the major form in cells not exposed to transmitter, while GTP γ S must wait until GDP dissociates from the G-protein.

G-protein activation by *t*-LHRH and muscarinic ACh receptors

The previous results provide evidence that a GTP-binding protein can suppress the M-current conductance. I further tested whether transmitter receptors suppress M-current through this pathway. The cells were dialyzed with internal solutions containing mixtures high in GTP and low in GTP γ S, to slow the spontaneous formation of GTP γ S bound G-proteins. Only if the G-protein's GTP-cycling rate is greatly accelerated by receptor activation will there be an appreciable build-up of GTP γ S-bound G-proteins.

Experiments were done using 2 different concentrations of GTP γ S (5 μM GTP γ S with 100 μM GTP; and 1 μM GTP γ S with 100 μM GTP), which should result in a 20- to 100-fold slowing of the rate of GTP γ S binding to G-proteins. Six cells were recorded from to examine the accumulation of irreversibly activated G-proteins, 2 with GTP γ S:GTP ratios of 1:100 and 4 with 5:100. With muscarine additions, 3 out of 3 applications produced irreversible suppression of M-current. For *t*-LHRH, 6 out of 6 applications caused irreversible suppression of M-current. Figure 5 shows the result of one experiment using 1 μM GTP γ S and 100 μM GTP in the internal solution. Dialysis for 10 min did not result in the loss of any M-current. Addition of 1 μM *t*-LHRH quickly suppressed the M-current by about 90%. This suppression accumulated until no M-current could be measured. When, after 3.5 min, the bath was washed out, only 28% of the M-current could be recovered; the rest was irreversibly lost.

Figure 5. Irreversible loss of M-current after receptor activation in cell with internal solution containing $1 \mu\text{M}$ GTP γS and $100 \mu\text{M}$ GTP. *A*, Control M-current after 11 min of dialysis. *B*, M-current reduced by more than 95% after 100 sec in $1 \mu\text{M}$ t-LHRH. *C*, reversal to 29% of control current with washout of t-LHRH after 400 sec.

Lack of IAP sensitivity

Studies of muscarinic activation of an atrial K channel showed that a pertussis toxin (IAP)-sensitive G-protein was responsible for the coupling of receptors to the channel (Pfaffinger et al., 1985). In addition, Higashida et al. (1986) report that IAP blocks a bradykinin-induced depolarization in NG108-15 cells, which they attributed to the suppression of an M-current-like conductance. Therefore, I examined the effect of IAP on the coupling of muscarinic ACh and t-LHRH receptors to the suppression of M-current.

Because frog cells cannot be held overnight at high temperatures, a 20-fold higher concentration of IAP was used than was required for chick atrial cells (Martin et al., 1985). Cells were incubated for either 24 hr ($n = 9$) or 48 hr ($n = 3$) at 25°C in culture medium containing $0.1 \mu\text{g/ml}$ IAP and then studied with pipettes containing the standard internal solution. After 24 hr in IAP, 5 out of 6 cells showed a normal suppression of M-current in response to bath application of $20 \mu\text{M}$ muscarine. The extent of suppression was equivalent to that in normal cells. The cell that failed to respond to muscarine did respond to t-LHRH, as was frequently found for cells not treated with IAP. When the bath was changed to $1 \mu\text{M}$ t-LHRH, the M-current was suppressed in 7 out of 8 cells. The average response to t-LHRH was a 75% reduction in M-current. The cell that failed to respond to t-LHRH had previously responded to muscarine. Thus, all cells responded either to muscarine or to t-LHRH.

Figure 6(left) shows the completely normal responses to $20 \mu\text{M}$ muscarine and to $1 \mu\text{M}$ t-LHRH in a cell treated for 48 hr with $0.1 \mu\text{g/ml}$ IAP. In 2 additional cells recorded after 48 hr in $0.1 \mu\text{g/ml}$ IAP, using an internal solution containing $20 \mu\text{M}$ GTP γS , the M-current was irreversibly suppressed. Apparently this response is not sensitive to IAP.

In order to confirm that IAP had modified the IAP-sensitive G-proteins with this treatment, I attempted to demonstrate a block of covalent labeling of G-proteins with ^{32}P -NAD and IAP by pretreatment of cells for 24 hr with IAP. However, as Figure 6(right) illustrates, this experiment was confounded by a lack of any IAP-labeled substrate in control frog sympathetic neurons. Figure 6(right) shows 2 autoradiographs from a labeling experiment on solubilized membrane proteins. The lanes *A–F* are a 15 min room temperature exposure, and *A'–F'* are a 2 hr exposure at -70°C . Lanes *A, B, A', B'* contain 120–150 $\mu\text{g/lane}$ of non-IAP-pretreated membrane proteins from frog sympathetic ganglion neurons; the others are control lanes using brain membrane proteins (200 $\mu\text{g/lane}$ frog brain membrane protein: *C, D, C', D'*; 100 $\mu\text{g/lane}$ rat brain membrane protein: *E, F, E', F'*), which demonstrate the efficiency of the labeling procedure and show that IAP can ADP-ribosylate proteins of frog brain.

The 15 min autoradiograph shows no visible bands in the sympathetic ganglion lanes; however, clear IAP-specific labeling has occurred using the frog and rat brain. Exposure for over 2 hr failed to reveal any IAP-specific bands from frog sympathetic ganglion neurons, while the signal from brain membrane labeling is profound. And, even after 2 d (not shown), there were no IAP-specific bands from frog sympathetic neuron membranes, suggesting, surprisingly, that these cells contain $<0.5\%$ as much IAP substrate per milligram membrane protein as frog brain and possibly none at all. This labeling experiment was attempted both on membranes and solubilized membrane proteins, using 120–200 μg of membrane protein per lane ($n = 5$), and it provides further evidence that an IAP-sensitive G-protein does not couple this response.

Other guanine nucleotide derivatives

I further characterized the coupling between receptors and the suppression of M-current by testing the effects of other guanine nucleotide derivatives. Two other guanine nucleotide derivatives commonly used to test for GTP-binding proteins are GPP(NH)P, a nonhydrolyzable GTP derivative that generally produces an irreversible activation of G-proteins, much like GTP γS (G-protein reviews: Gilman, 1987; Stryer and Bourne, 1986), and GDP βS , a GDP derivative that is a competitive inhibitor of many G-protein-mediated processes (Eckstein et al., 1979).

Figure 7 summarizes the effect of different internal guanine nucleotides on the receptor/G-protein-mediated suppression of M-current in 4 cells. The response of the M-current addition and washout of $1 \mu\text{M}$ t-LHRH was recorded at -35 mV . In a normal experiment using $100 \mu\text{M}$ GTP inside (Fig. 7*A*), M-current was almost completely suppressed by t-LHRH. After washout of the t-LHRH, the M-current increased to a level greater than before (over-recovery). With dialysis solution containing $100 \mu\text{M}$ GDP βS and $20 \mu\text{M}$ GTP (Fig. 7*B*), the M-current was only partly suppressed by t-LHRH, suggesting that GDP βS may act as a weak competitive inhibitor to GTP. However, even in cells dialyzed with $10 \mu\text{M}$ GDP βS alone, both muscarine and t-LHRH were able to suppress M-current, although not as well as in cells dialyzed with GTP. The average suppression of M-current by $1 \mu\text{M}$ t-LHRH was 88% with GTP but only 50% with GDP βS . Surprisingly, some M-current remains irreversibly suppressed after reversal in all cells dialyzed with GDP βS . This loss of M-current accumulated with multiple agonist additions and during longer applications of agonist, making evaluation of the inhibitory effect difficult.

With $100 \mu\text{M}$ GPP(NH)P inside (Fig. 7*C*), M-current was again almost completely suppressed ($94 \pm 2\%$; $n = 4$) by t-LHRH. However, unlike experiments with GTP, M-current reversed to

Figure 6. *Left*, Normal responses in cell pretreated at 25°C for 48 hr with 0.1 μ g/ml IAP. Standard internal solution containing GTP. *A–C*, M-current was reduced by 69% in 20 μ M muscarine and reversed to 103% of control with 0.5 μ M atropine. *D* and *E*, Subsequent addition of 1 μ M t-LHRH reduced the M-current by 80%; current reversed to 112% of control after the bath was washed out with Ringer's. *Right*, Lack of IAP-sensitive G-proteins in frog sympathetic neurons. *A–F*, 15 min room temperature autoradiograph of a SDS electrophoresis gel from a solubilized membrane protein IAP/ 32 P-NAD labeling experiment. *A'–F'*, Same experiment but 2 hr exposure at -70°C . Lanes containing membrane proteins labeled with IAP/ 32 P-NAD are *A,A'*; *C,C'*; and *E,E'*. Lanes for nonspecific labeling containing only 32 P-NAD are *B,B'*; *D,D'*; and *F,F'*. The sources and amounts of solubilized membrane protein for the different lanes are as follows: *A,B,A',B'*, 120–150 μ g of frog sympathetic neuron membrane protein; *C,D,C',D'*, 200 μ g of frog brain membrane protein; *E,F,E',F'*, 100 μ g of rat brain membrane protein. Absence of labeling in lanes *A,A'* indicates that frog sympathetic neurons lack IAP-sensitive G-proteins.

only about 60% of its control level after washout of t-LHRH compared with the control reversal of 130%. Similar results were found with muscarine. The suppression with 100 μ M muscarine was $69 \pm 7\%$ compared with $52 \pm 3\%$ using GTP, and there was reversal to $78 \pm 5\%$ of its control level.

Finally, as described earlier, with 1 μ M GTP γ S and 100 μ M GTP inside (Fig. 7*D*), M-current was again suppressed by transmitter, with the majority of the current irreversibly lost by the time t-LHRH was washed off.

Discussion

This paper demonstrates the whole-cell voltage-clamp recording of M-current and its control by muscarine and t-LHRH in isolated, dialyzed frog sympathetic ganglion neurons. M-current was recorded stably in cells dialyzed with the standard internal solution. The standard solution contained the protease inhibitor leupeptin because in early experiments before leupeptin was added, M-current was suppressed poorly, or not at all, after adding muscarine. The reason for this is unclear, but active intracellular proteases could potentially damage many steps in the transduction pathway.

In contrast to the stability of M-current in cells dialyzed with the standard internal solution, M-current is lost in cells dialyzed without ATP. I found that functional M-channels require hydrolyzable ATP by demonstrating that substitution of ATP with

the nonhydrolyzable derivative APP(NH)P did not prevent loss of M-current. The rapid loss of M-current in the absence of ATP implies that an active cycle of phosphorylation and dephosphorylation of the critical site(s) exists in these cells. This presents a possible new mechanism for receptor-mediated suppression of M-current, either through inhibition of the enzyme responsible for the constitutive phosphorylation and/or by activation of a phosphatase directed at these site(s).

Some characteristics of muscarinic and t-LHRH suppression of M-current were different from those reported in previous microelectrode work. First, M-current was less sensitive to muscarine and more sensitive to t-LHRH than has been reported. This quantitative difference in activity may be due to species differences, differences in cell preparation, a difference in receptor number, or a susceptibility of muscarinic receptors to whole-cell dialysis. It does not seem to reflect a difference in coupling pathways, as later experiments demonstrate the similarities in the pathways used by muscarinic and t-LHRH receptors. Second, responses to both agonists showed some slow desensitization during sustained applications. The desensitization was induced by the agonist since the first responses to the drugs were quite similar over a wide range of dialysis times (3–30 min). Third, M-current grew by 30% following the addition and washout of either t-LHRH or muscarine, a process I have named over-recovery. Apparently, M-current channels are not functioning maximally either at the moment of breakthrough

Figure 7. Effect of different guanine nucleotides on the suppression of M-current by 1 μ M t-LHRH and subsequent recovery. Each experiment is from a different cell dialyzed for over 8 min with the indicated internal solution before addition of t-LHRH. Current amplitudes are normalized to that before t-LHRH addition. Internal solutions as follows: *A*, 100 μ M GTP; *B*, 100 μ M GDP β S + 20 μ M GTP; *C*, 100 μ M GPP(NH)P; *D*, 100 μ M GTP + 1 μ M GTP γ S.

or after the small initial rise that occurs with dialysis alone. A cycle of agonist stimulation seems to initiate intracellular processes (phosphorylations?) that may bring a reserve pool of "silent" channels into play or increase the open probability of channels that are already functioning. This raises the possibility that there may be some agonist/receptor combinations that would enhance M-current without suppressing it. The M-current's amplitude was generally not increased further by multiple applications of agonists; therefore, during over-recovery the M-current may reach an absolute maximum amplitude for the cell. Finally, a new K-current, I_{KP} , is activated by both muscarine and t-LHRH. This current was not seen at all unless the membrane potential was prepulsed more negative than -70 mV, probably because of tonic inactivation at more depolarized potentials.

These results suggest that, in addition to suppression of M-current, agonists initiate other processes in sympathetic neurons, including activation of the over-recovery mechanism, initiation of the slow desensitization process, and activation of the I_{KP} current. Since these processes have not been described in microelectrode studies, it is possible that they have been augmented here by dialysis.

My experiments provide functional evidence that a GTP-binding protein is involved in muscarinic and t-LHRH control of M-current. Dialysis of GTP γ S, AlF_4^- , or F^- into cells causes the suppression of M-current, thus mimicking transmitter application. The fact that both GTP γ S and AlF_4^- activate G-pro-

teins suggests that G-protein activation is sufficient to reproduce the effect of muscarine and t-LHRH on the M-current. Although dialysis without added GTP did not prevent the muscarinic suppression of M-current, muscarinic ACh and t-LHRH transmitter receptors do appear to activate this G-protein. Both receptors produced irreversible loss of M-current after dialysis with low-GTP γ S, high-GTP internal solutions. For any given application of agonist, the amount of M-current that was irreversibly lost depended on 3 factors: (1) the ratio of GTP γ S to GTP, (2) the size of the initial response to transmitter, and (3) the length of time the transmitter was on the cell. This irreversible loss probably reflects the fraction of activated G-proteins that have bound GTP γ S rather than GTP. I have modeled the results from these experiments to obtain estimates of the functional properties expected for the G-protein (Pfaffinger, 1987). I estimate that the time constant for the G-protein's GTPase is 2.5–7 sec. Furthermore, I estimate that only 1–5% of the G-proteins are activated without agonist, thus insuring a good signal after receptor activation. These results are consistent with the requirements for G-protein involvement in the physiological suppression of M-current. Hence, transmitters most likely control the M-current by controlling the activity of this G-protein; however, since no actual G-protein was isolated, other interpretations are possible.

The hypothesis of a G-protein intermediate can be conclusively proven only when the identity of this putative G-protein is known. Based on my experiments, the G-protein responsible

for the suppression of M-current has several distinctive characteristics. First, since responses to either muscarine or t-LHRH were insensitive to pertussis toxin (IAP), the G-protein is an IAP-insensitive G-protein. This suggests that the G-protein controlling M-current in frog sympathetic neurons is different from the IAP-sensitive G-protein that is reported to control an M-like conductance in NG108-15 cells (Higashida and Brown, 1986; Higashida et al., 1986). Surprisingly, these sympathetic neurons actually lack any G-proteins that can be ADP-ribosylated by IAP. Second, muscarine and t-LHRH responses had the following behavior with different internal guanine nucleotides: Both were irreversible in the presence of GTP γ S, only partially irreversible with GPP(NH)P, and reversible to a level greater than before the response with GTP. GDP β S weakly blocked responses to either transmitter; thus, GDP β S is probably a poor competitive inhibitor for this G-protein, although the inhibitory action of GDP β S was complicated by the poor reversal of responses after dialysis with GDP β S.

My results define a potency series: GTP γ S $\gg$ GPP(NH)P $>$ GTP $>$ GDP β S. This series should be characteristic for the G-protein that is involved in the control of M-current. Biochemical studies of IAP-insensitive G-protein in other systems have measured similar potency series: GTP γ S 50 times more potent than GPP(NH)P in the activation of phospholipase C in 7315c tumor cells (Aub et al., 1986). GTP γ S stimulates polyphosphoinositide hydrolysis in GH₃ cells, whereas GPP(NH)P is almost ineffective (Lucas et al., 1985). Muscarinic receptors interact with an IAP-insensitive G-protein in 1321N1 human astrocytoma cell line (Evans et al., 1985). The carbachol binding affinity is modulated by guanine nucleotides, with the following potency series: GTP γ S $>$ GPP(NH)P = GTP = GDP. These results, along with my own, suggest that IAP-insensitive G-proteins have a much higher affinity for GTP γ S than GPP(NH)P. GPP(NH)P has a lower affinity than GTP γ S for other G-proteins; however, GPP(NH)P is very effective in activating the IAP-sensitive G-protein that controls a K channel in atrial cells (Breitwieser and Szabo, 1985). Future studies will attempt to establish the precise identity of this G-protein using purified G-proteins or antibodies to G-proteins.

Two additional conclusions are suggested from my results. First, muscarinic ACh receptors and LHRH receptors appear to activate the same G-protein, which then leads to suppression of M-current; therefore, convergence of these 2 pathways probably occurs at the first step in the pathway. Second, the lack of IAP-sensitive G-proteins in frog sympathetic neurons suggests that negative control of cAMP, if it occurs in these cells, is not via G_i but rather through some other pathway, such as phosphodiesterase activation.

Finally, the question of how M-channel suppression is finally achieved remains open. Three potential mechanisms are supported by these and other studies. The first mechanism is the direct suppression of M-channels by a G-protein in a manner analogous to that proposed for the activation of a K channel in atrial cells (Pfaffinger et al., 1985; Kurachi et al., 1986). This mechanism receives some circumstantial support from the sustained loss of M-current in sympathetic neurons dialyzed with G-protein activators, suggesting a fairly tight coupling between G-protein activation and M-current suppression. The second mechanism is activation of the phosphatase and/or suppression of the kinase that controls phosphorylation of the site required by M-current. The third mechanism is the activation of phospholipase C, as suggested by Higashida and Brown (1986). Al-

though their work points towards an IAP-sensitive G-protein, in some other systems, IAP-insensitive G-proteins control phospholipase C. Such an IAP-insensitive pathway has been implicated for muscarinic ACh receptors (Evans et al., 1985; Masters et al., 1985; Dunlop and Larkins, 1986) and possibly LHRH receptors (Naor et al., 1986). However, experiments by Malenka et al. (1986) on an M-like conductance of hippocampal neurons have suggested that protein kinase C does not control this current. Therefore, future experiments will be directed at determining the role of protein kinase C in the control of M-current in frog sympathetic neurons.

References

- Adams, P. R., D. A. Brown, and A. Constanti (1982a) M-currents and other potassium currents in bullfrog sympathetic neurons. *J. Physiol. (Lond.)* 330: 537-572.
- Adams, P. R., D. A. Brown, and A. Constanti (1982b) Pharmacological inhibition of the M-current. *J. Physiol. (Lond.)* 332: 223-262.
- Adams, P. R., D. A. Brown, and S. W. Jones (1983) Substance P inhibits the M-current in bullfrog sympathetic neurones. *Br. J. Pharmacol.* 79: 330-333.
- Aub, D. L., E. A. Frey, R. D. Sekura, and T. E. Cote (1986) Coupling of the thyrotropin-releasing hormone receptor to phospholipase C by a GTP-binding protein distinct from the inhibitory or stimulatory GTP-binding protein. *J. Biol. Chem.* 261: 9333-9340.
- Bigay, J., P. Deterre, C. Pfister, and M. Chabre (1985) Fluoroaluminates activate transducin-GDP by mimicking the γ -phosphate of GTP in its binding site. *FEBS Lett.* 181: 181-185.
- Breitwieser, G. E., and G. Szabo (1985) Uncoupling of cardiac muscarinic and β -adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* 317: 538-540.
- Brown, D. A., and P. R. Adams (1980) Muscarinic suppression of a novel voltage-sensitive K⁺ current in a vertebrate neurone. *Nature* 283: 673-676.
- Cook, D. L., and N. Hales (1984) Intracellular ATP directly blocks K channels in pancreatic B-cells. *Nature* 311: 271-273.
- Dunlop, M. E., and R. G. Larkins (1986) Muscarinic agonist and guanine nucleotide activation of polyphosphoinositide phosphodiesterase in isolated islet-cell membranes. *Biochem. J.* 240: 731-737.
- Eckstein F., D. Cassel, H. Levkovitz, M. Lowe, and Z. Selinger (1979) Guanosine T'-O-(2-Thiodiphosphate): An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *J. Biol. Chem.* 254: 9829-9834.
- Eiden, L. E., E. Loumaye, N. Sherwood, and R. L. Eskay (1982) Two chemically and immunologically distinct forms of luteinizing hormone-releasing hormone are differentially expressed in frog neural tissue. *Peptides* 3: 323-327.
- Evans, T., M. W. Martin, A. R. Hughes, and T. K. Harden (1985) Guanine nucleotide-sensitive, high affinity binding to muscarinic cholinergic receptors of 1321N1 astrocytoma cells is insensitive to pertussis toxin. *Mol. Pharmacol.* 27: 32-37.
- Gilman, A. G. (1987) G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56: 615-649.
- Halliwel, J. V., and P. R. Adams (1982) Voltage-clamp analysis of muscarinic excitation in hippocampal-neurons. *Brain Res.* 250: 71-92.
- Halvorsen, S. W., and N. M. Nathanson (1984) Ontogenesis of physiological responsiveness and guanine nucleotide sensitivity of cardiac muscarinic receptors during chick embryonic development. *Biochemistry* 23: 5813-5821.
- Higashida, H., and D. A. Brown (1986) Two polyphosphatidylinositol metabolites control two K⁺ currents in a neuronal cell. *Nature* 323: 333-335.
- Higashida, H., R. A. Streaty, W. Klee, and M. Nirenberg (1986) Bradykinin-activated transmembrane signals are coupled via N_o or N_i to production of inositol 1,4,5-trisphosphate, a second messenger in NG108-15 neuroblastoma-glioma hybrid cells. *Proc. Natl. Acad. Sci. USA* 83: 942-946.
- Jan, Y. N., C. W. Bowers, D. Branton, L. Evans, and L. Y. Jan (1983) Peptides in neuronal function: Studies using frog autonomic ganglia. *Cold Spring Harbor Symp. Quant. Biol.* 48: 363-374.
- Jones, S. W., P. R. Adams, M. J. Brownstein, and J. E. Riviers (1984)

- Teleost luteinizing hormone-releasing hormone: Action on bullfrog sympathetic ganglia is consistent with role as neurotransmitter. *J. Neurosci.* 4: 420–429.
- Kurachi, Y., T. Nakajima, and T. Sugimoto (1986) On the mechanism of activation of muscarinic K^+ channels by adenosine in isolated atrial cells: Involvement of GTP-binding proteins. *Pfluegers Arch.* 407: 264–274.
- Lindau, M., and J. M. Fernandez (1986) IgE-mediated degranulation of mast cells does not require opening of ion channels. *Nature* 319: 150–153.
- Lucas, D. O., S. M. Bajjalieh, J. A. Kowalchuk, and T. F. J. Martin (1985) Direct stimulation by thyrotropin-releasing hormone (TRH) of polyphosphoinositide hydrolysis in GH_3 cell membranes by a guanine nucleotide-modulated mechanism. *Biochem. Biophys. Res. Commun.* 132: 721–728.
- Malenka, R. C., D. V. Madison, R. Andrade, and R. A. Nicoll (1986) Phorbol esters mimic some cholinergic actions in hippocampal pyramidal neurons. *J. Neurosci.* 6: 475–480.
- Martin, J. M., D. D. Hunter, and N. M. Nathanson (1985) Islet activating protein inhibits physiological responses evoked by cardiac muscarinic acetylcholine receptors. Role of guanosine triphosphate binding proteins in regulation of potassium permeability. *Biochemistry* 24: 7521–7525.
- Masters, S. B., M. W. Martin, T. K. Harden, and J. H. Brown (1985) Pertussis toxin does not inhibit muscarinic receptor mediated phosphoinositide hydrolysis or calcium mobilization. *Biochem. J.* 227: 933–937.
- Matteson, D. R., and C. M. Armstrong (1986) Properties of two types of calcium channels in clonal pituitary cells. *J. Gen. Physiol.* 87: 161–182.
- Naor, Z., A. Azrad, R. Limor, H. Zakut, and M. Lotan (1986) Gonadotropin-releasing hormone activates a rapid Ca^{2+} -independent phosphodiester hydrolysis of polyphosphoinositides in pituitary gonadotrophs. *J. Biol. Chem.* 261: 12506–12512.
- Pfaffinger, P. J. (1987) Role of GTP-binding proteins in receptor-mediated control of potassium channels. Ph.D. Thesis, University of Washington, University Microfilms, Ann Arbor, MI.
- Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille (1985) GTP-binding proteins couple cardiac muscarinic receptors to a K -channel. *Nature* 317: 536–538.
- Pobiner, B. F., E. L. Hewlett, and J. C. Garrison (1985) Role of N_1 in coupling angiotensin receptors to inhibition of adenylate cyclase in hepatocytes. *J. Biol. Chem.* 260: 16200–16209.
- Sims, S. M., J. J. Singer, and J. V. Walsh (1985) Cholinergic agonists suppress a potassium current in freshly dissociated smooth-muscle cells of the toad. *J. Physiol. (Lond.)* 367: 503–529.
- Sternweis, P. C., and A. G. Gilman (1982) Aluminum: A requirement for activation of the regulatory component of adenylate cyclase by fluoride. *Proc. Natl. Acad. Sci. USA* 79: 4888–4891.
- Stryer, L., and H. R. Bourne (1986) G-proteins: A family of signal transducers. *Annu. Rev. Cell Biol.* 2: 391–419.