The Journal of Neuroscience, October 1987, 7(10): 3306–3316

Dependence of an Adenosine-Activated Potassium Current on a GTP-Binding Protein in Mammalian Central Neurons

Laurence O. Trussell and Meyer B. Jackson

Department of Biology, University of California, Los Angeles, Los Angeles, California 90024

Neurons in hippocampal and striatal cell cultures respond to adenosine with an inhibitory potassium current. This response disappears during whole-cell patch-clamp recording in which the cell is filled with minimal saline. We have found that this loss of sensitivity to adenosine can be prevented by including 100 μM GTP in the patch electrode filling solution. GDP is less effective than GTP in supporting the adenosine response, while GMP has little, if any, effect. Treatments known to inhibit GTP-binding proteins (G-proteins) block the adenosine-activated potassium current: The adenosine response is inhibited by including poorly metabolized analogs of guanine nucleotides along with GTP in the recording electrode. Diphosphate and triphosphate analogs appear to achieve this effect through different mechanisms. The adenosine response is also blocked by incubating cultures in islet-activating protein (pertussis toxin), an inhibitor of a class of G-protein. Thus, our data implicate a G-protein in the activation of a potassium current by adenosine. Intracellular ATP can increase the effectiveness of GMP, GDP, or low concentrations of GTP, suggesting that even during internal dialysis, neurons can maintain GTP levels through phosphotransferase reactions. Intracellular ATP also appears to suppress an outward current that is different from the adenosine-activated current. Raising intracellular cAMP levels either with bath-applied forskolin or by including a cAMP analog in the recording electrode did not alter the adenosine response. These results indicate that a G-protein is involved in the coupling between the adenosine receptor and a potassium channel, and that this coupling is not mediated by cAMP.

Adenosine is now recognized as a potent modulator of electrical activity throughout the nervous system. Studies of its action have focused on the types and distribution of adenosine receptors, of ionic mechanisms, and of the modulation of adenylate cyclase activity (Phillis and Wu, 1981; Daly et al., 1983; Premont et al., 1983; Snyder, 1985). Adenosine can inhibit the electrical activity of neurons through the activation of a potassium conductance and the inhibition of a calcium conductance (Segal, 1982; Greene and Haas, 1985; Trussell and Jackson, 1985; Dolphin et al., 1986; MacDonald et al., 1986). However, it remains unclear what cytoplasmic components are required for adenosine receptor binding to be transduced into an electrophysiological response.

One approach to this kind of problem is to try to mimic receptor activation by adding to the extracellular medium agents that stimulate or inhibit second messenger systems. More recently, intracellular dialysis or perfusion techniques, including the whole-cell patch-clamp, have offered a powerful alternative to the study of cytoplasmic regulation of ion channels because they allow one to manipulate directly the intracellular milieu while recording electrophysiological responses (Kostyuk et al., 1975; Hamill et al., 1981). During recordings made using these techniques, reductions in intracellular metabolite concentrations would be expected as small, rapidly diffusing molecules are quickly equilibrated with the patch electrode filling solution. Indeed, studies of voltage- or neurotransmitter-gated ion channels using intracellular dialysis have often been plagued by the apparent disappearance or rundown of ionic currents during the experiments (Kostyuk et al., 1981; Byerly and Hagiwara, 1982; Trussell and Jackson, 1985). By replacing the lost cytoplasmic materials, this approach ultimately resulted in the identification of biochemical components that can regulate calcium currents (Byerly and Yazejian, 1986; Chad and Eckert, 1986).

In this report we use the whole-cell patch-clamp technique to investigate the cytoplasmic requirements of the response of cultured neurons to adenosine. We have previously shown that in cultured mouse striatal neurons, adenosine activates an inwardly rectifying potassium conductance (Trussell and Jackson, 1985). We now find that adenosine responses in cultured hippocampal neurons are essentially the same as those in cultured striatal neurons, and are probably identical to adenosine responses of pyramidal cells in hippocampal slices (Segal, 1982; Greene and Haas, 1985). Furthermore, the adenosine-activated potassium current disappears during a whole-cell patch-clamp recording. This loss of sensitivity appears to reflect the loss of intracellular GTP. Our results suggest that the adenosine receptor acts on a potassium channel through a G-protein and that adenylate cyclase modulation is not critically involved in this effect. A presentation of this result has appeared in abstract form (Trussell and Jackson, 1986).

Materials and Methods

Cultures were prepared from either striatum or hippocampus from 18 d mouse embryos, as described by Trussell and Jackson (1985). The tissue was treated for 30 min at 37°C with 0.25% trypsin in isosmolar calcium-free saline and then triturated. Cells were plated at a density of 10^6 cells/35 mm collagen-coated dish (Falcon) in minimum essential medium (MEM), with 10% horse and 10% fetal calf serum. In later experiments, Falcon "Primaria" dishes were used without collagen coating. After 5 d, the medium was changed to MEM containing 10% horse

Received Jan. 26, 1987; revised Apr. 7, 1987; accepted Apr. 23, 1987.

We would like to thank Drs. David Armstrong, Anton Hermann, Jeanne Nerbonne, Michael Rogowski, and Mr. Jerrel Yakel for helpful discussion and comments, and Linda Attardo and Huda Dethlefs for preparation of the cultures. This work was funded by NIH Fellowship NS 07821 (to L.O.T.) and HD 5958 and NS 21908 (to M.B.J.).

Correspondence should be addressed to Laurence Trussell at his present address: Washington University School of Medicine, Box 8108, Dept. of Anatomy and Neurobiology, 660 So. Euclid Ave., St. Louis, MO 63110.

serum, 10 μM fluorodeoxyuridine, and 50 μM uridine. Two days later the medium was changed to MEM with 10% horse serum, which was thereafter changed twice weekly. Cultures were used 1–4 weeks after plating.

For recordings, cells were bathed at 34°C in a solution containing (in mM) 138 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 4 KCl, and 10 HEPES-Na, pH 7.2. The basic patch electrode filling solution was 120 K-gluconate, 1 $MgCl_2$, 2.38 $CaCl_2$, 5 BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, tetrapotassium salt), 20 sucrose (for osmotic balance), and 10 HEPES, pH 7.2. All intracellular nucleotide solutions described in Results used this solution as a base to which nucleotides were added. All experiments using nonhydrolyzable guanine nucleotide analogs also included 1 mM ATP in the recording electrode. The free Ca^{2+} concentration in this solution was computed to be 97 nM, assuming an absolute affinity constant for BAPTA of 9.35×10^6 M^{-1} and pH independence (Tsien, 1980). Experiments in which the free calcium was varied from 100 to less than 1 nM or the added magnesium was varied from 0 to 4 mM did not obviously affect the adenosine response.

Cells were voltage-clamped by the tight-seal whole-cell recording technique (Hamill et al., 1981) with an EPC-7 patch-clamp (List/Medical Systems). Responses to adenosine were generally recorded at a membrane potential of −65 mV. Aluminum silicate patch electrodes had resistances of 1–4 MΩ. After establishment of an intracellular recording, the access resistance (series resistance from the pipette into the cell) was usually between 3 and 10 MΩ; recordings were rejected if the access resistance exceeded 14 MΩ during the experiment. A low access resistance was necessary to maintain a low diffusion barrier between the electrode and the cell interior and to observe a consistent loss of adenosine sensitivity with control solutions. Small-tipped electrodes of borosilicate glass were used as noted below to lower the rate of diffusion. These electrodes had resistances between 10 and 20 MΩ, and produced access resistances of 25 to over 100 MΩ.

Adenosine (Sigma) and forskolin (Calbiochem), dissolved in bath medium, were applied focally to the experimental cell through puffer pipettes. Adenosine was pressure-ejected with 100 msec pulses at 2–5 psi (1 psi = 6.89 kPa). The final concentration of adenosine on the cells' surface was probably not the same as that in the puffer because of the need to have only brief responses. Pertussis toxin (List Biochemicals) was dissolved in sterile distilled water to a final concentration of 100 μg/ml and stored for up to 2 d at 0–4°C. Purine nucleotides were obtained from Boehringer Mannheim and Sigma.

Results

Response to adenosine

Focal application of 50 μM adenosine to striatal neurons produces an outward current that reverses near the expected equilibrium potential for potassium of −90 mV (Fig. 1*A*; Trussell and Jackson, 1985). Indeed, the reversal potential for this response varies linearly with the logarithm of the external potassium concentration with a slope of −55 mV, in excellent agreement with the Nernst relation for a potassium-selective conductance (Fig. 1*C*). This current is also characterized by a marked inward rectification: At potentials positive to the rest potential, the current–voltage relation curves downward (Fig. 1*B*; Trussell and Jackson, 1985). In cultures from the hippocampus, adenosine activates a conductance whose current–voltage relation is indistinguishable from the adenosine response of striatal neurons (Fig. 1*B*). The presence of the adenosine-activated outward current in cultured hippocampal neurons is consistent with studies made on the effects of adenosine in the hippocampal slice preparation (Segal, 1982; Greene and Haas, 1985).

Loss of adenosine sensitivity

A striking characteristic of the response to adenosine in both hippocampal and striatal neurons is that the response disappears during recordings made with *low*-resistance patch electrodes (see Materials and Methods). Figure 2*A* shows records of the outward current induced by adenosine in a hippocampal neuron and its decay during whole-cell recording. When a low-resistance electrode filled with a control solution (see Materials and Methods) is used for recording, the response decays to less than 50% of its initial value within 10 min, and is almost gone by 20 min. Figure 2*B* illustrates the time course of the loss of sensitivity with pooled data from many cells (solid circles). Previous experiments have suggested that the loss of the adenosine response does not reflect a receptor desensitization process because it is dependent on the size of the patch electrode tip, rather than the frequency of agonist application (Trussell and Jackson, 1985). Pooled data obtained using *high*-resistance electrodes (see Materials and Methods; Fig. 2*B*, open circles) reveal that, under these recording conditions, the response to adenosine is not attenuated by repeated application. Indeed, when high-resistance electrodes are employed, no apparent decay of the response is observed even with *continuous* bath-application of adenosine for over 5 min (not shown). Thus, loss of sensitivity to adenosine appears to depend on the rate of exchange of the intracellular medium with the electrode solution. We will refer to this phenomenon of loss of sensitivity to adenosine as "washout," to distinguish it from true desensitization and to reflect the effects observed below with guanine nucleotides. γ-Aminobutyric acid responses (Trussell and Jackson, 1985), glutamate responses (D. Finch and M. B. Jackson, unpublished observations), and a fast excitatory serotonin response (Yakel et al., 1986) remain constant with time. Thus, washout is not likely to result from a general cytolytic process. Finally, washout is unlikely to be the result of the loss of voltage-gated calcium currents (Byerly and Hagiwara, 1982), as the adenosine-activated potassium current is not dependent on calcium influx (Trussell and Jackson, 1985; see Materials and Methods).

Throughout this study, no differences were observed between hippocampal and striatal neurons with regard to washout and the regulation of the adenosine current. We will therefore present data for both brain regions without distinction.

Intracellular action of guanine nucleotides

Because of the well-documented action of adenosine on adenylate cyclase activity (Snyder, 1985), it might be expected that washout occurs because cAMP, or other soluble cytoplasmic factors that are required for adenylate cyclase activity or protein phosphorylation, diffuses out of the cell into the patch electrode. However, we found that the addition of 100 μM GTP to the electrode solution could prevent washout of the adenosine response (Fig. 2*C*). Figure 2*D* shows the average behavior of cells for which patch electrodes contained either 50 (open circles) or 100 μM (solid circles) GTP. With 100 μM GTP, the response decayed by less than 20%. With 50 μM GTP, the response declined to a plateau at approximately 50% of its initial value. While the protective effect of GTP was variable from cell to cell, in some cases we observed virtually no attenuation of the response for over 45 min of recording with electrode access resistances as low as 3 MΩ. By contrast, 1 mM ATP did not prevent washout (Fig. 2*D*, triangles), indicating a genuine selectivity for the guanine nucleotide, and also that the loss of sensitivity is not simply due to a metabolic rundown.

Figure 3 shows a dose–response relation for the protective effect of GTP on the adenosine response obtained with low-resistance electrodes. Adenosine response amplitudes were measured after 18 min of whole-cell recording, which is sufficient time for equilibration between the cell and the electrode (see

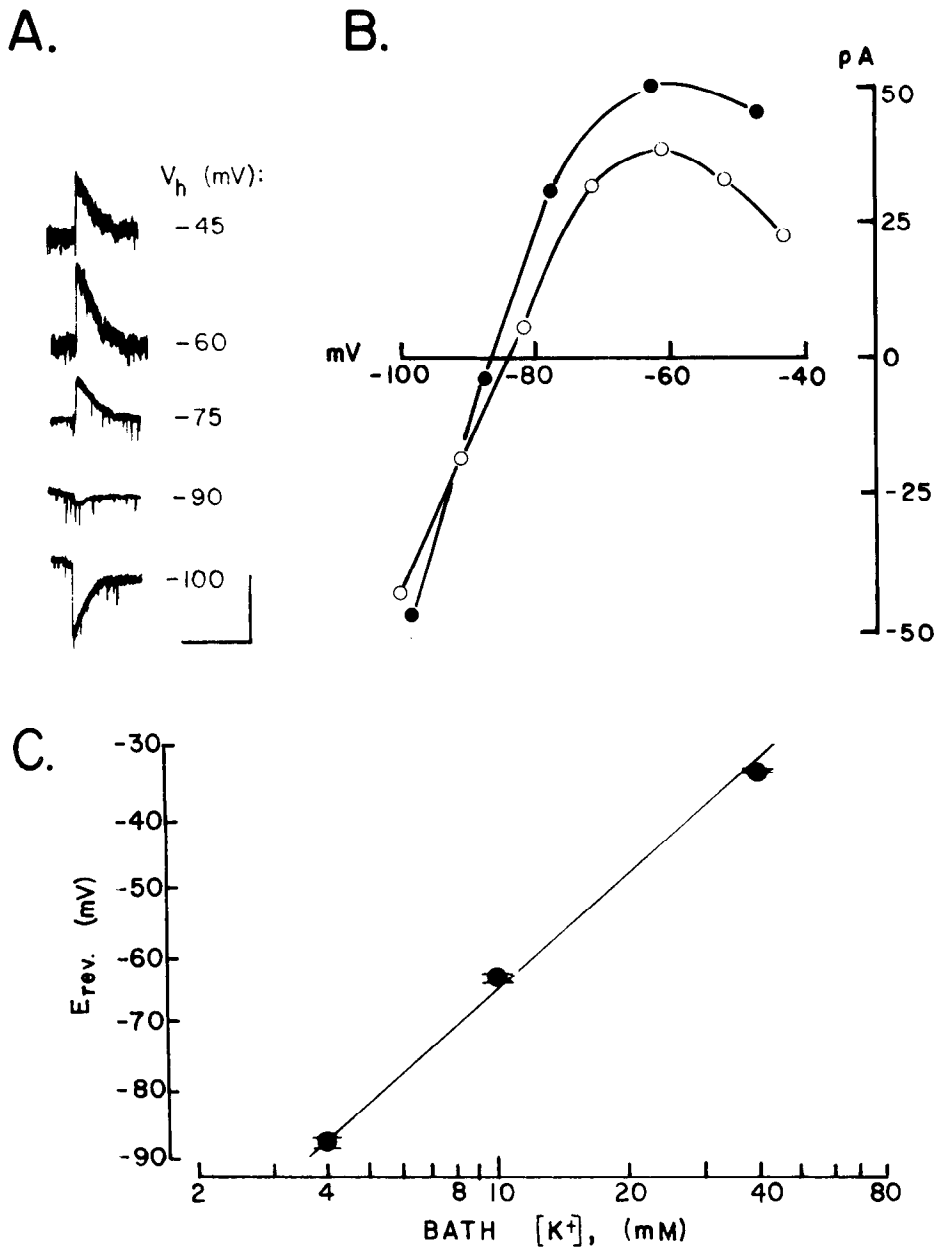

Figure 1. Characteristics of the adenosine-activated current. *A*, Chart recordings of adenosine responses at the indicated holding potentials. Adenosine (50 μM) was pressure-ejected near the cell, as described in Materials and Methods. Calibration bars, 40 sec and 50 pA. *B*, Adenosine-activated current was taken from records such as those in *A* and plotted as a function of holding potential for hippocampal (●) and striatal (○) neurons. The response reverses close to the calculated potassium reversal potential of −90 mV. *C*, Reversal potential for the adenosine-activated current versus the concentration of potassium in the bathing media, where reversal potentials were obtained by interpolating through 0 current on plots such as *B*. Each average is from 2–4 cells. Potassium chloride replaced sodium chloride to elevate extracellular potassium. The slope of the best-fit line shown is −55 mV.

Fig. 2). The half-maximal protective effect of GTP is about 50 μM, which is in the physiological range for intracellular GTP (Henderson and Paterson, 1973). Interestingly, the maximal protection is, on average, only about 80%. While this might be explained in part by a change in the driving force for potassium during intracellular dialysis, it may be that part of this small, "residual" washout is due to buildup of GDP (see below). It should be noted that greater variability is observed with 1 mM GTP than with lower concentrations. Indeed, marked washout was sometimes observed when this unphysiological dose was used.

Also included in Figure 3 is a comparison of the protective effects of 100 μM GDP and GMP to that of GTP. It is clear that the ability of the nucleotides to prevent washout drops markedly as the nucleotide contains fewer phosphates. Additionally, in 2 experiments in which 1 mM of guanosine alone or cyclic guanosine monophosphate (cGMP) was included in the recording electrode the adenosine response completely washed out.

GMP, by itself, is unable to support the response to adenosine. However, we observed that both intracellular GMP and adenosine monophosphate (AMP) were able to antagonize the protective effects of intracellular GTP. With 400 μM GMP and 100 μM GTP in the recording electrode, the adenosine response decayed to 28 ± 24% (±SD; $n = 3$) of its initial amplitude after 18 min, as compared to 81 ± 9% ($n = 4$) in cells filled with 100 μM GTP alone. Similarly, with 400 μM AMP and 100 μM GTP in the electrode, the responses in 2 cells decayed to 18 and 34%. The inhibition produced by the monophosphate nucleotides was significant at the 5% level, using Student's *t* test.

Intracellular action of guanine nucleotide analogs

A requirement for GTP is likely to reflect the involvement of one of the G-proteins (Gilman, 1984; Roof et al., 1985). Such proteins play a role in a number of membrane receptor-mediated responses, including physiological responses to putative neurotransmitters (Pfaffinger et al., 1985; Aghajanian and Wang,

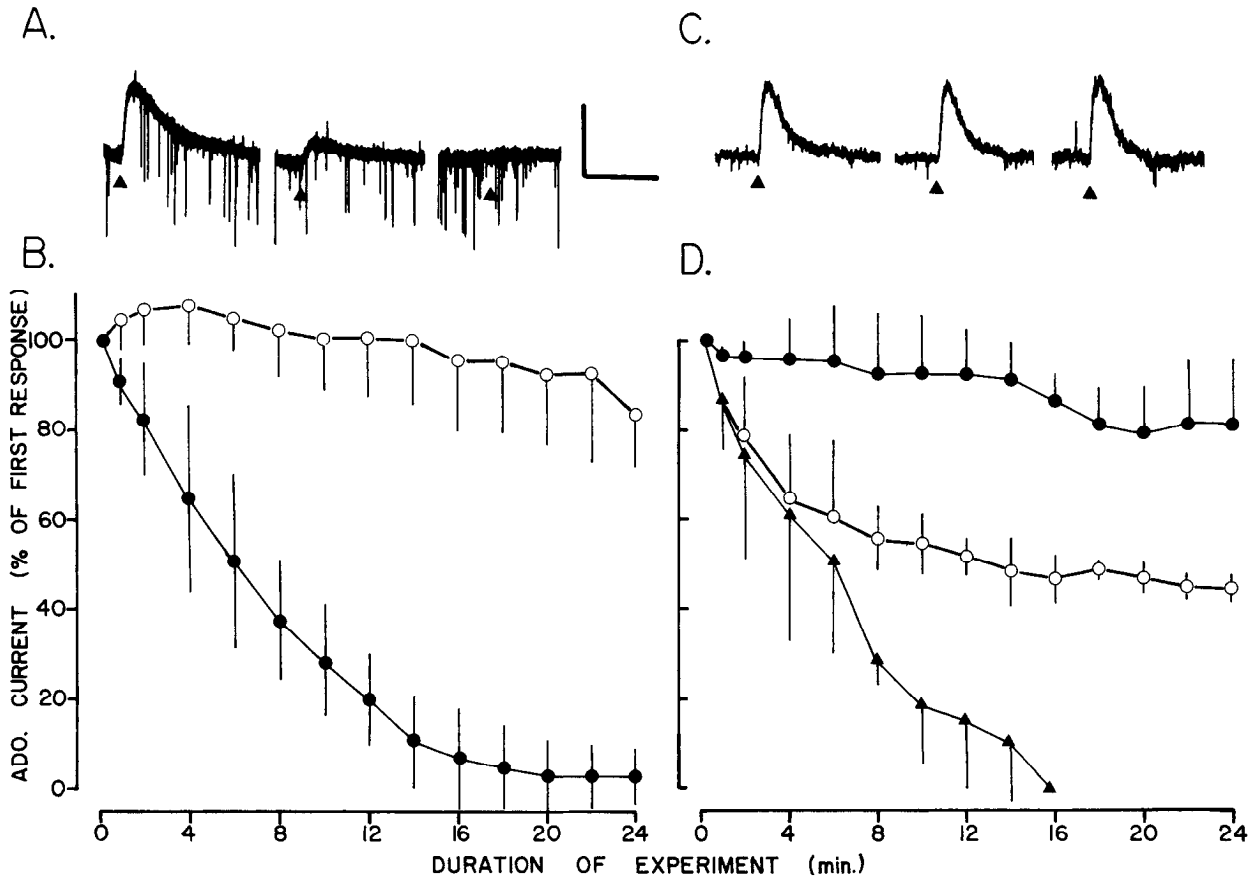

Figure 2. Time course of changes in adenosine-activated current during whole-cell patch-clamp recordings. Cells were held at −65 mV. *A, C,* Chart recordings of responses to pressure-ejected 50 μM adenosine made 1, 10, and 20 min after initiating the whole-cell recording. Recording electrodes contained, for *A,* control solution and, for *C,* control solution plus 100 μM GTP. Calibration bar, 25 pA (*A, C*), 20 sec (*A*), and 40 sec (*C*). *B, D,* Variations in adenosine responses over time. For each cell, adenosine response amplitudes, measured at each time point, were normalized to the first response recorded for that cell; these data were then averaged for different cells. *Vertical lines* show the standard deviations. *B,* Filling low-resistance patch electrodes with control solution (●, 9 cells); filling high-resistance patch electrodes with control solution (○, 8 cells). *D,* Data obtained using low-resistance electrodes filled with control solution plus 100 μM GTP (●, 4 cells), 50 μM GTP (○, 3 cells), or 1 mM ATP (▲, 6 cells).

1986; Andrade et al., 1986; Holz et al., 1986; Kurachi et al., 1986). Available evidence suggests that G-proteins couple membrane receptors to other cellular functions by a process requiring the hydrolysis of GTP. Guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) is a poorly metabolized analog of GDP that inhibits G-proteins, presumably by preventing the binding of GTP (Eckstein et al., 1979). Figure 4, *A, B,* shows that the adenosine response decays rapidly with time when 150 μM GDPβS plus 100 μM GTP are present in the recording electrode. (ATP, 1 mM, was also present in experiments using these analogs. See section below on ATP effects.) The addition of GDPβS increases the rate of washout approximately 4-fold over that seen with control filling solution, despite the presence of GTP (compare to Fig. 2*B*). These data suggest that endogenous GTP is involved in the adenosine response and that it can be displaced by GDPβS. Increasing the ratio of GTP to GDPβS appears to reduce the rate and extent of washout (Fig. 4*B*) in a manner consistent with a competitive blocking action at a GTP binding site.

A similar loss of sensitivity to adenosine was observed when electrodes contained poorly hydrolyzed analogs of GTP. The presence of guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) or of guanylyl-imidodiphosphate (GPP(NH)P) in the recording electrodes also results in a rapid decay in adenosine responses (Fig. 4, *C, D*). These data also reveal that increasing the ratio of GTP to GTP analog reduced the rate and, at least for GPP(NH)P, the extent of washout. The action of these compounds in our experiments is entirely consistent with their known behavior on G-proteins: Both substances can bind very tightly to the GTP binding site and, because they are not hydrolyzed, induce the G-protein to become continuously active (Gilman, 1984). Thus, addition of adenosine under these conditions would not be expected to cause further activation of the G-protein or produce a discrete electrophysiological response. A similar action of GPP(NH)P on a potassium channel activated by acetylcholine in heart muscle has been observed by Breitwieser and Szabo (1985).

One would expect that if the GTP analogs were inhibiting the adenosine response by activating the G-protein independently of agonist, then the potassium current normally activated by adenosine would be turned on in a constitutive manner. In fact, experiments with GTPγS were always associated with an increasing outward holding current and a marked increase in membrane current noise (Fig. 5*A*). A similar effect was observed with GPP(NH)P (not shown). The recording in Figure 5*A* is striking both for the rapidity of onset of this steady outward current and for the degree to which it exceeds the amplitude of the first adenosine response. Quantitation of this effect was difficult because of the variability among different cells in the rate

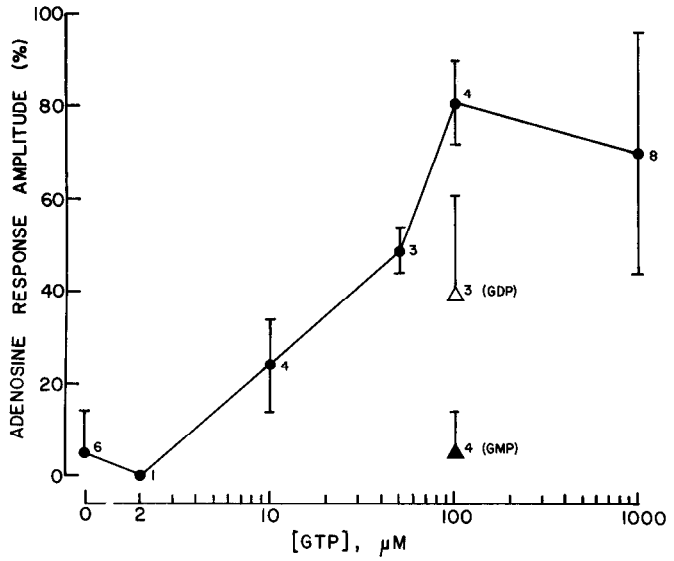

Figure 3. Dose–response for the prevention of washout of the adenosine response by GTP. Ordinate is the amplitude of the adenosine response after 18 min of whole-cell recording, expressed as a percentage of the response obtained immediately after beginning the recording. Number of cells averaged for each point is shown near each *symbol.* Recording electrodes contained the indicated concentration of GTP, except for the *open and closed triangles,* which are from electrodes containing 100 μM GDP and GMP, respectively.

of onset and magnitude of this steady current. The following procedure was used to normalize these variables: Because the cells were held near their initial resting potential, the value of the holding current at the start of the experiment was defined as zero. The value of the holding current just before each application of adenosine was then divided by the amplitude of the *initial* adenosine response in order to see by how much the changing holding current exceeded the adenosine current. In Figure 5*B,* this measure of the change in holding current is compared to the relative amplitude of the adenosine current at different points in time (rather than to the time course of the recording itself). For comparison, we also plot these parameters for cells filled with GDPβS.

Figure 5*B* illustrates several points about the action of these analogs. First, the increase in the holding current observed with GTPγS is directly proportional to the loss of adenosine sensitivity, as would be expected if GTPγS were opening those channels normally activated by adenosine. Second, GDPβS induces loss of adenosine sensitivity, but has no striking effect on the holding current, consistent with its inhibitory effect on G-proteins. Finally, the current activated by GTPγS exceeds the current initially activated by adenosine by as much as 3-fold. Preliminary dose–response relations indicated that 50 μM adenosine was indeed a saturating dose for this adenosine receptor, and that the brief pressure puffs used to deliver adenosine produce a response that is greater than 75% of the maximal response. Therefore, the current induced by GTPγS cannot be entirely accounted for by the activation of channels functionally coupled to the adenosine receptor, but must result from the behavior of other channels as well (see Discussion).

It is possible that some of the channels that contribute to the GTPγS-induced outward holding current are of a different kind from those described in Figure 1, and perhaps some of the outward current results from the *reduction* of a steady inward current. In practice, it proved difficult to obtain current–voltage relations before and after the appearance of the steady outward current because of its rapid onset. However, if those channels opened by adenosine are also opened by GTPγS, the outward holding current should be reduced by channel-blocking agents that reduce the adenosine current. The current produced by adenosine was unaffected by either 10 mM tetraethylammonium or 1 mM cesium, applied extracellularly (not shown). Other workers have shown that the adenosine-induced hyperpolarization of pyramidal neurons is immune to 4-aminopyridine (Segal, 1982; Greene and Haas, 1985). We found, however, that 1 mM extracellular barium was an effective blocker of the adenosine response. Figure 6*A* shows that pressure-ejection of barium-containing solutions markedly reduced the response to adenosine. Bath-application of barium completely blocked the response. Figure 6, *B, C,* shows that the development of the outward current and apparent loss of adenosine sensitivity are accompanied by an increasing effect of barium application on the holding current. As the holding current moved outward, adenosine produced smaller outward currents and barium produced larger inward currents. Thus, these data indicate that GTPγS may act to inhibit adenosine sensitivity by irreversibly activating those potassium channels normally controlled by adenosine, probably through the constitutive activation of a G-protein. However, we do not know whether adenosine itself is required to initiate this activation, as in the case of the muscarinic current in the heart (Breitwieser and Szabo, 1985).

Action of pertussis toxin

A number of G-proteins have been identified in mammals. Two of these, G_s and G_i, are characterized by their role in the coupling of hormone receptor binding to the stimulation and inhibition, respectively, of adenylate cyclase (Gilman, 1984). Another G-protein, G_o, is found in bovine brain. It appears not to be coupled to adenylate cyclase and its function is not known at present (Roof et al., 1985). Both G_i and G_o are ADP-ribosylated by pertussis toxin (Gilman, 1984; Sternweis and Robishaw, 1984). Pertussis toxin inactivates G_i; the consequences of ADP-ribosylation of G_o are not yet known. Recently, it has been shown that pertussis toxin abolishes the inhibitory effect of adenosine on glutamate release (Dolphin and Prestwich, 1985). We find that this toxin also blocks the adenosine-activated potassium current: Treatment of hippocampal cell cultures with pertussis toxin for 5 hr dramatically reduced the ability of the cells to respond to adenosine (Fig. 7). This treatment had no detectable effect on the cells in terms of their superficial appearance, membrane potential, or input resistance. Treatment of cultures with heat-inactivated pertussis toxin had no effect on adenosine responses (Fig. 7). These results suggest that the activation of a potassium current by adenosine depends on a substrate of pertussis toxin, which is most likely either G_i or G_o.

Actions of ATP

As noted above, 1 mM ATP in the electrode was not, by itself, able to prevent washout of the adenosine response. However, when 1 mM ATP was included *with* GTP in the patch electrode, the adenosine response was markedly potentiated, especially at lower concentrations of GTP. This effect is illustrated in Figure 8, which compares steady-state adenosine responses for different GTP concentrations, with and without ATP. However, ATP

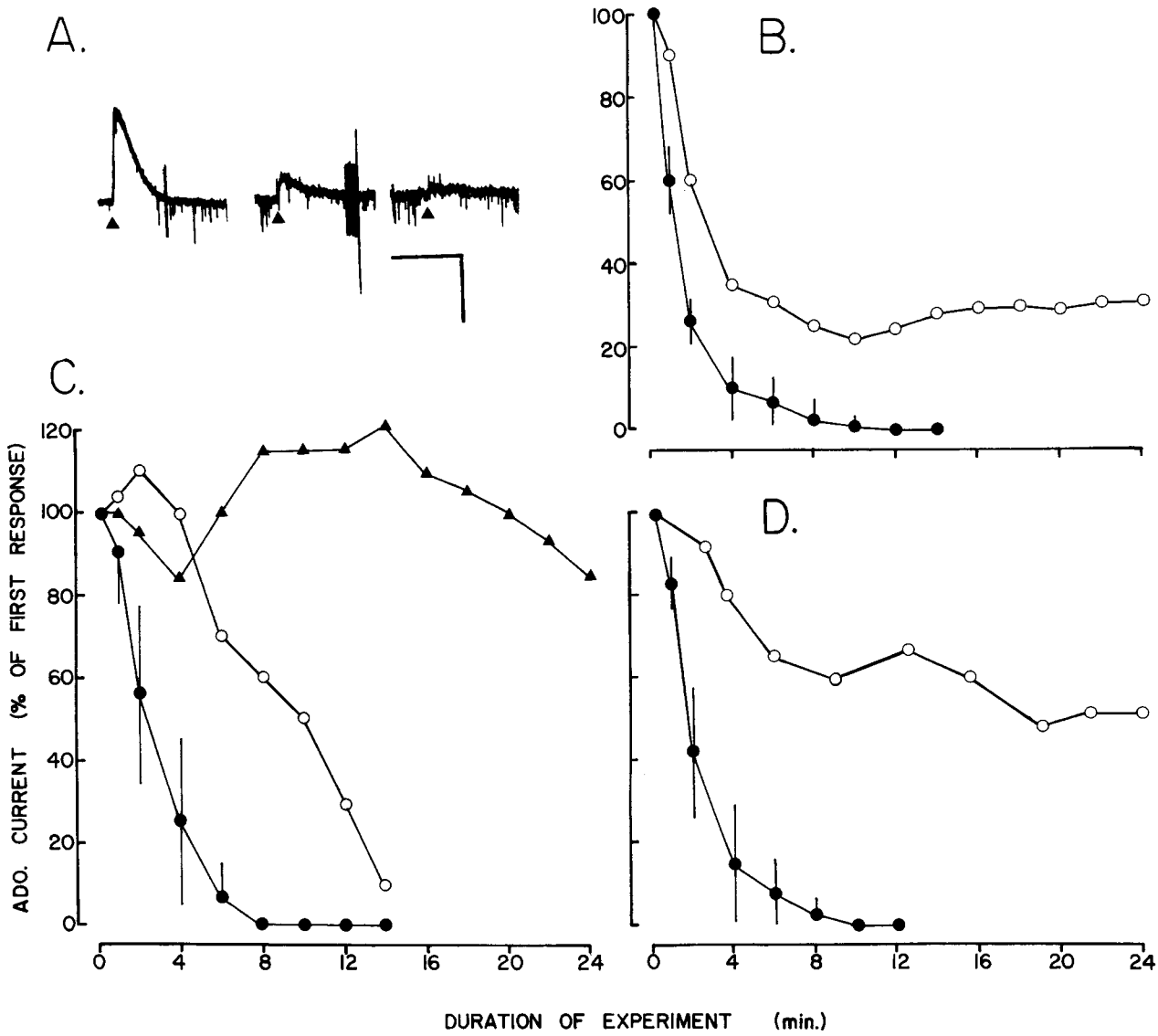

Figure 4. Washout in the presence of GTP plus guanine nucleotide analogs. *A,* Chart recorder traces of washout time course, showing traces at 1, 4, and 8 min after the start of the recording. The recording electrode was filled with control solution plus 100 μM GTP and 150 μM GDPβS. Calibration bar, 40 sec, 25 pA. *B* and *D,* Time course of washout for averaged cell responses. *Vertical bars* represent standard deviation. *B,* Low-resistance electrodes contained control solution with 100 μM GTP and 150 μM GDPβS (●, 5 cells), or with 100 μM GTP and 50 μM GDPβS (○, 1 cell). *C,* Recording electrodes contained control solution with 100 μM GTP and 20 μM GTPγS (●, 4 cells), 5 μM GTPγS (○, 1 cell), or 1 μM GTPγS (▲, 1 cell). *D,* Recording electrodes contained control solution with 100 μM GTP and 500 μM GPP(NH)P (●, 3 cells) or 100 μM GTP and 100 μM GPP(NH)P (○, 1 cell). All recordings were made with 1 mM ATP in the electrode as well.

was also observed to potentiate adenosine responses from cells filled with 100 μM GDP or even GMP (Fig. 8). The ability of ATP to potentiate the effects of guanine nucleotides appears to depend on the donation of a terminal phosphate, since 1 mM β,γ-methylene ATP, a poorly hydrolyzable ATP analog, could not substitute for ATP in potentiating the effects of 50 μM GTP (Fig. 8).

An additional effect was observed with intracellular ATP. In cells internally dialyzed with either control saline or control saline plus GTP, an outward movement of the holding current was observed, beginning within a few minutes after starting whole-cell recording, and stabilizing within 10 min. This current was not observed in cells studied with 1 mM ATP in the recording electrode. Figure 9*A* illustrates recordings with 10 μM GTP alone (top trace) and with 10 μM GTP plus 1 mM ATP in the electrode (bottom trace). While this outward current could be pronounced in the absence of ATP, it was unaffected by the presence of GTP. Figure 9*B* shows the magnitude of this outward current after 10 min of recording for cells with ATP (solid bars), without ATP (open bars), and with low and high concentrations of GTP. It is clear that concentrations of GTP that are able to prevent washout of the adenosine response (see Fig. 3) do not prevent the development of this shift in holding current or affect the action of ATP. Thus, the adenosine-activated potassium current is regulated by mechanisms that are distinct from those that control this ATP-blocked current. This outward current is quite variable in magnitude, and its sharp onset, as illustrated in Figure 9*A*, requires an electrode–cell access resistance of less than 10 MΩ. While we have not studied it further, it seems doubtful that this effect is related to improvement in

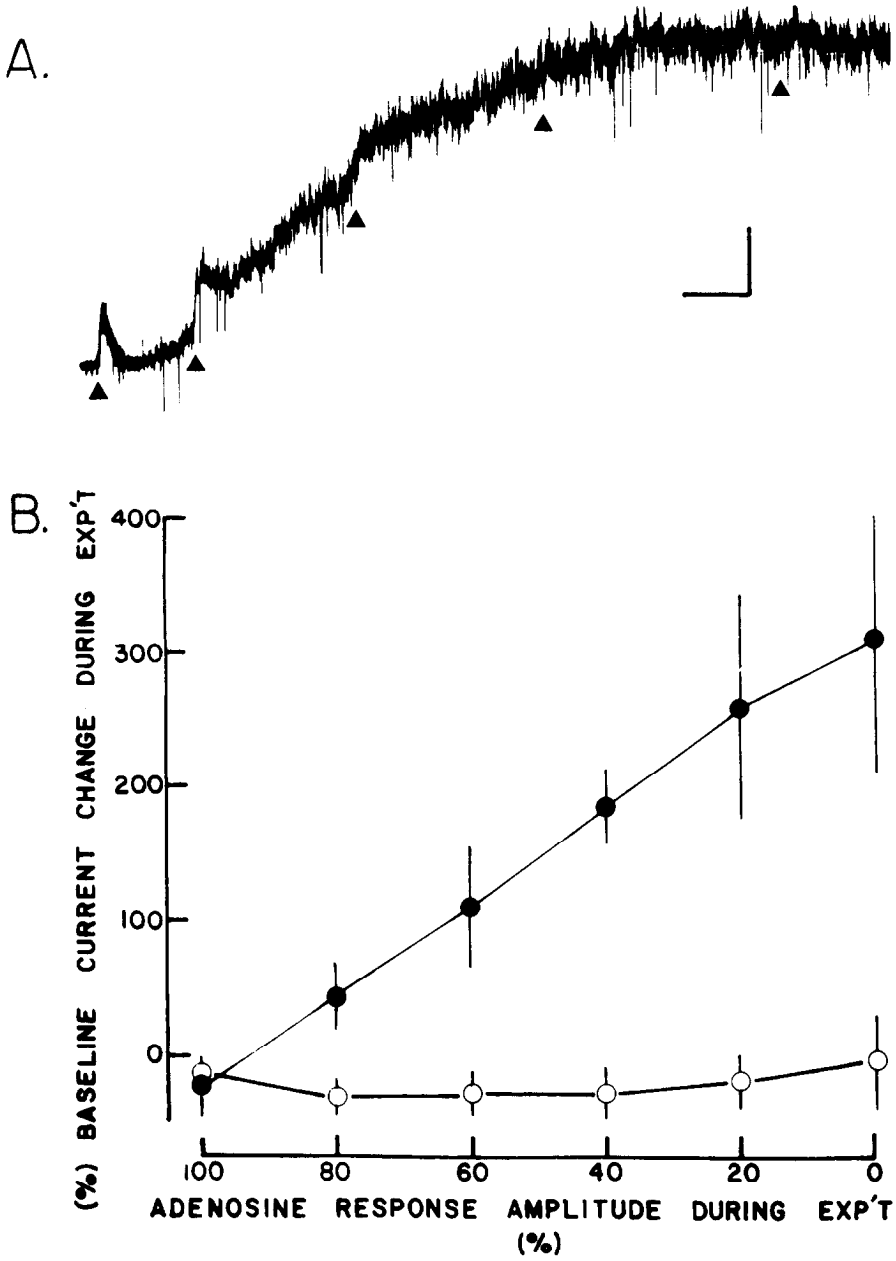

Figure 5. Effects of intracellular GTPγS on the baseline holding current. The cells were held at −65 mV. *A*, Chart recording of the onset of the GTPγS-activated outward holding current and the parallel decay in the adenosine-evoked current. The record began simultaneously with the rupture of the membrane patch. Adenosine was pressure-ejected at the points indicated by *triangles*. Recording electrodes contained 100 μM GTP, 20 μM GTPγS, and 1 mM ATP. *B*, Correlation between development of the outward holding current and the reduction in adenosine-activated current. Ordinate is the holding current (change in baseline from time zero) divided by the amplitude of the first adenosine response and multiplied by 100. The first adenosine response was measured within 10–20 sec after patch rupture. Abscissa is the adenosine response measured at a given point in time, divided by the first adenosine response in the recording and multiplied by 100 (as shown in Figs. 2 and 4). Holding current change was always measured just before applying adenosine. *Closed circles*, average of 4 cells filled with the same solution as in A. *Open circles*, 5 cells filled with 100 μM GTP, 150 μM GDPβS, and 1 mM ATP. *Vertical lines*, standard deviations.

intracellular calcium buffering by ATP (Byerly and Yazejian, 1986), as the outward current is observed with electrode solutions containing 5 mM BAPTA and 0 added calcium. It is perhaps analogous to the ATP-inhibited potassium current observed in cardiac and pancreatic cells (Cook and Hales, 1984; Kakei et al., 1985). It may be that the conductance regulated by ATP is sensitive to GTPγS, and thus contributed to some of the holding current shift described earlier in cells filled with this analog. However, this seems unlikely, as the effect of GTPγS on the ATP-inhibited channel of pancreatic cells is shared by GTP, GDP and GDPβS, and its actions are reduced by ATP (Dunne and Petersen, 1986).

Involvement of cAMP

Since the addition of GTP alone is sufficient to maintain the adenosine-activated potassium current, we questioned whether the well-documented modulation of adenylate cyclase by adenosine is a necessary step in the activation of the potassium conductance. This seemed doubtful because cAMP and its precursor, ATP, are just as likely to be lost from the cell as GTP, and yet they are not needed in the patch electrode to maintain the response to adenosine. However, we undertook a more thorough examination of the role of cAMP in the adenosine response by adding the adenylate cyclase activator forskolin (Seamon et al., 1981), at a concentration of 50 μM, to the bathing medium (Fig. 10). To prevent the loss of cytoplasmic components during the recording, these experiments were carried out with small-tipped patch electrodes (see Fig. 2) filled with control solution. The ratio of the response in the presence of forskolin to the response in the absence of forskolin was 1.02 ± 0.11 (mean ± SD; 6 cells), indicating that the response was not altered by this treatment. The application of forskolin results in a slow inward current in these cells, which is similar to the response to norepinephrine (Yakel et al., 1986), also an activator of adenylate

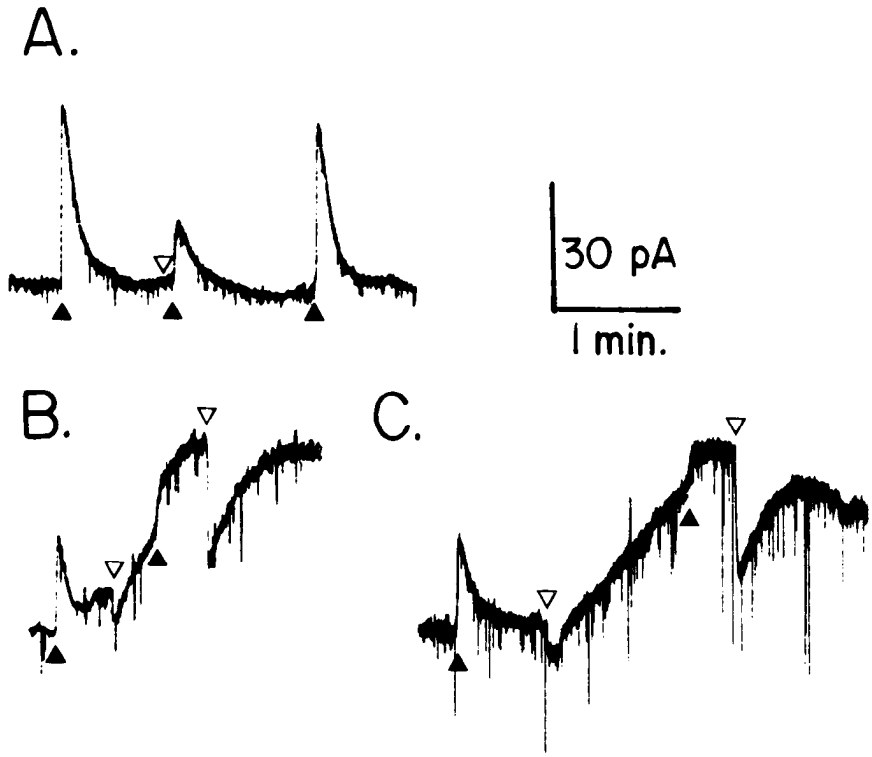

Figure 6. Actions of barium on adenosine-activated currents in voltage-clamped hippocampal neurons. *A,* Solid triangles show time of pressure-application of 50 μM adenosine. Barium chloride (1 mM in bath solution) was applied by diffusion from a pipette placed near the cell at the times marked by the *open triangle.* The barium pipette was removed immediately before adenosine application to avoid interfering with the application. The cell was recorded with a high-resistance electrode. *B, C,* In 2 different cells recorded with 20 μM GTPγS, 100 μM GTP, and 1 mM ATP in a low-resistance electrode, responses to 50 μM adenosine (*solid triangles*) grow weaker, while responses to 1 mM barium (*open triangles*) grow stronger during the development of the holding current shift. Initiation of whole-cell recording coincides with start of traces, as shown.

cyclase in cultured mouse striatal neurons (Premont et al., 1983). Thus, we are confident that forskolin is effective as a means of elevating intracellular cAMP in our experiments.

Forskolin does not block or mimic adenosine's action, suggesting that changes in cAMP levels do not mediate the adenosine response. However, one might argue that adenosine activation of the G-protein inhibitory to adenylate cyclase (G_i) could itself inhibit forskolin's action (Hudson and Fain, 1983). Data such as those shown in Figure 10 do not reveal any tendency for adenosine to actually reverse the effects of forskolin. We nevertheless carried out additional experiments in which 1 mM 8-bromo cAMP, together with 100 μM GTP and 1 mM ATP, were added to a low-resistance recording electrode. This approach assures that the cyclic nucleotide is directly introduced into the cell at a high level and is not degraded. The results were not supportive of cAMP as a second messenger in the adenosine

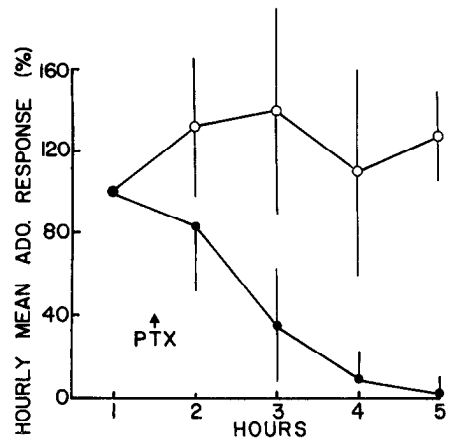

Figure 7. Effect of islet-activating protein (pertussis toxin) on the adenosine-activated potassium current. Ordinate shows the average response to adenosine of different cells in periods of 1 hr. Averages are expressed relative to the average response in the first hour of recording. In 2 experiments pertussis toxin was added directly to the bath from a stock solution (see Materials and Methods) after the first hour to give a final concentration of 3.3 or 0.5 μg/ml (*solid circles*). Control data (*open circles*) include one experiment in which nothing was added to the dish and one experiment in which 5 μg/ml of pertussis toxin that had been held at 90°C for 15 min was added. Cells were maintained at 34°C during toxin incubation. Each mean includes data from 3–8 cells.

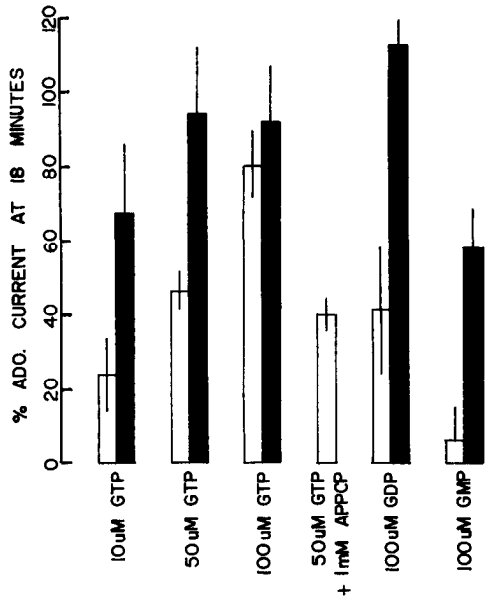

Figure 8. Effect of intracellular ATP on the ability of guanine nucleotides to maintain the adenosine response. Data shown for the adenosine responses of 2–9 cells averaged after 18 min of whole-cell recording; *lines through bars* are the standard deviations. Guanine nucleotide additions to the control electrode solution are as indicated *below the bars. Filled bars* are for electrode solutions that also contained 1 mM ATP.

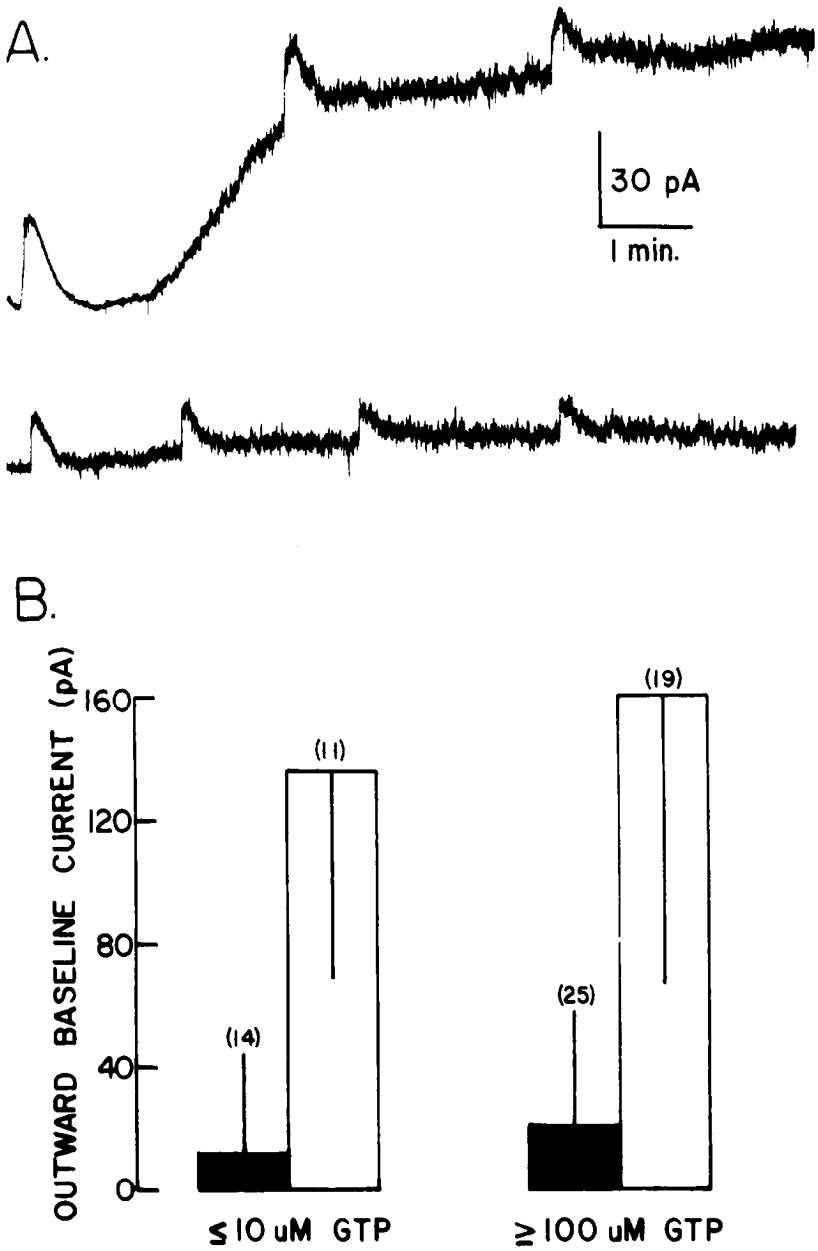

Figure 9. Intracellular ATP inhibits an outward current. *A,* Chart recording of the beginning of whole-cell recording for 2 cells from the same culture dish. In the *upper trace,* the electrode contained 10 μM GTP and 0 ATP, while in the *lower trace* the electrode contained 10 μM GTP and 1 mM ATP. Note that adenosine responses decay to about the same level. *B,* Magnitude of the outward currents that develop after 10 min of whole-cell recording with cells held at −65 mV. *Solid bars* are recordings with 1 mM ATP, while *open bars* are recordings in its absence. Electrodes also contained GTP concentrations as shown. *Numbers above bars* are the number of cells averaged for each category.

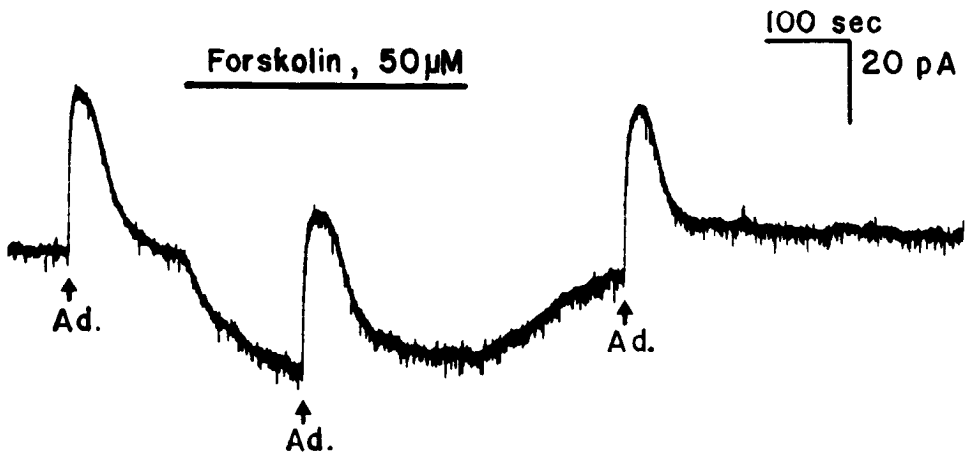

Figure 10. The adenosine responses before, during, and after application of 50 μM forskolin. Forskolin alone produces an inward current upon which the adenosine response is superimposed. Forskolin was applied in a slow stream by a wide-barrel pipette. A high concentration of adenosine (1 mM) was applied in a brief (20 msec) pulse from a second, much smaller pressure pipette to avoid interrupting the application of forskolin.

response. In these experiments the response was measured as soon as an intracellular recording was established, and again 18 min later. The average ratio of the second response to the first was 1.18 ± 0.35 (mean ± SD; 4 cells). The ratio for control experiments with nucleotide triphosphates alone was 0.93 ± 0.14 (8 cells) and was not significantly different from the mean for cells with the cAMP analog. As with forskolin and norepinephrine treatment, a slow inward current developed as the cell filled with 8-bromo cAMP. This inward current reached a plateau within approximately 5 min, supporting the validity of comparing the initial response with that seen after 18 min.

Discussion

G-proteins are now recognized as a common link in the coupling of many sorts of membrane receptors to a variety of intracellular processes, including adenylate cyclase activation, ion channel modulation, phospholipid breakdown, and the release of intracellular calcium stores (Gilman, 1984; Cockcroft and Gomperts, 1985; Holz et al., 1986; Ueda et al., 1986). In heart muscle, the coupling of adenosine and muscarine receptors to an inwardly rectifying potassium channel by G-proteins has been convincingly demonstrated (Pfaffinger et al., 1985; Kurachi et al., 1986), and it now appears that the coupling is maintained even in isolated heart cell membrane patches (Kurachi et al., 1986). The present study is the first to extend this mechanism to adenosine action in neurons of the mammalian central nervous system, directly documenting the requirement of the cells for GTP via intracellular dialysis techniques.

We have also demonstrated that cAMP is not likely to be an intermediary in this response. While direct introduction of 1 mM 8-bromo cAMP did appear to enhance slightly the response to adenosine, this dose is over 4 orders of magnitude larger than the K_m for kinase activation by cAMP (Nestler and Greengard, 1984). Thus, if modulation of cAMP levels were a necessary step in the activation of the potassium channel by adenosine, 8-bromo cAMP should have reduced the responsiveness of the cells to adenosine. If a second messenger of another sort is involved, then it must be resistant to washout during intracellular dialysis. The actual consequences of adenylate cyclase modulation by adenosine remain obscure. Perhaps its role lies in affecting a calcium conductance or calcium-dependent potassium conductance (Madison and Nicoll, 1986), in modulating the more acute effects of adenosine and other transmitters on a long-term basis, or in nonelectrophysiological functions that are independent of adenosine's inhibitory effects. Consistent with our findings, it has been reported that an extracellular application of 8-bromo cAMP does not mimic the response to adenosine in hippocampal slices (Madison and Nicoll, 1986). If the adenosine response is not in fact mediated by any diffusible second messenger, then one would expect to see adenosine-activated channels in isolated membrane patches, with GTP on the intracellular face, as was observed in the heart by Kurachi et al. (1986). This differs sharply from a potassium conductance activated by adenosine in the *Xenopus* oocyte, in which cAMP appears to be a true second messenger in the response (Lotan et al., 1985).

The potentiation of the effects of guanine nucleotides by ATP was a surprising finding, not predicted from our model of adenosine's effects on a G-protein. The inability of β,γ-methylene ATP to substitute for ATP in producing this effect suggests that ATP acts by donating a terminal phosphate. In the case of GTP potentiation, it is possible that ATP acts by participating in a phosphorylation of a protein that is involved in the activation of the potassium channels. However, a more straightforward scheme is that ATP acts through one of the common phosphotransferase reactions (Henderson and Paterson, 1973), converting GDP and GMP back to GTP. GDP buildup very near the G-protein by hydrolysis of GTP could inhibit G-protein activity by preventing GTP binding. Indeed, GDP and GTP have very similar affinities for G-proteins (Bokoch et al., 1984). Low doses of GTP could be made more effective by recycling GDP that was hydrolyzed by G-protein activity. This hypothesis would also explain how ATP can render GDP and GMP very effective agents in preventing washout, even though they are not able to activate G-proteins. It assumes, however, that local concentrations of metabolites within the cell may not be the same as those in the electrode during intracellular dialysis. This might be expected if such compounds build up in the cell more quickly than they diffuse into the electrode.

Recent evidence suggests that the type of potassium current that is affected by adenosine here may prove to be coupled to many different transmitter receptors in the nervous system. For example, a neuronal potassium current showing inward rectification can be activated by adenosine, baclofen, enkephalin, norepinephrine, and serotonin (Gahwiler and Brown, 1985; Newberry and Nicoll, 1985; North and Williams, 1985; Trussell and Jackson, 1985; Yakel et al., 1986). Furthermore, it appears likely that, as with adenosine, the serotonin and baclofen responses of hippocampal neurons are dependent on GTP and G-proteins (Andrade et al., 1986). This raises the possibility that a similar conductance and transduction mechanism is associated with a variety of different receptors on the same cell. It will be of interest to determine whether adenosine receptors share individual ion channels with other neurotransmitter receptors or instead have their own pool of channels. In the case of serotonin and baclofen receptors, additivity experiments indicate that the receptors do share the same conductance (Andrade et al., 1986). It therefore seems likely that receptors for many of these agents, including adenosine, may be functionally coupled to the same ion channel. Variation in the density of these different receptors might lead to cases where one transmitter opens only a fraction of the channels controlled by another transmitter. In this context, the ability of GTPγS to activate a current much larger than that of adenosine is understandable, as the analog could act on the entire pool of G-protein-regulated potassium channels. These observations suggest that both short- and long-term regulation of the responsiveness of neurons to adenosine may be intimately related not only to adenosine itself, but to the activity of other neuromodulatory substances as well.

References

Aghajanian, G. K., and Y.-Y. Wang (1986) Pertussis toxin blocks the outward currents evoked by opiate and α_2-agonists in locus coeruleus neurons. Brain Res. *371:* 390–394.

Andrade, R., R. C. Malenka, and R. A. Nicoll (1986) A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science *243:* 1261–1265.

Bokoch, G. M., T. Katada, J. K. Northup, M. Ui, and A. G. Gilman (1984) Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. J. Biol. Chem. *259:* 3560–3567.

Breitwieser, G. E., and G. Szabo (1985) Uncoupling of muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analog. Nature *317:* 538–540.

Byerly, L., and S. Hagiwara (1982) Calcium currents in internally

perfused nerve cell bodies of *Limnea stagnalis*. J. Physiol. (Lond.) *322:* 503–528.

Byerly, L., and B. Yazejian (1986) Intracellular factors for the maintenance of calcium currents in perfused neurones from the snail, *Lymnea stagnalis*. J. Physiol. (Lond.) *370:* 631–650.

Chad, J. E., and R. Eckert (1986) An enzymatic mechanism for calcium current inactivation in dialyzed *Helix* neurones. J. Physiol. (Lond.) *378:* 31–52.

Cockcroft, S., and G. D. Gomperts (1985) Role of guanine nucleotide binding protein in the action of polyphosphoinositol phosphodiesterase. Nature *314:* 534–536.

Cook, D. L., and N. Hales (1984) Intracellular ATP directly blocks K^+ channels in pancreatic β-cells. Nature *311:* 271–273.

Daly, J. W., Y. Kuroda, J. W. Phillis, H. Shimizu, and M. Ui (1983) *Physiology and Pharmacology of Adenosine Derivatives,* Raven, New York.

Dolphin, A. C., and S. A. Prestwich (1985) Pertussis toxin reverses adenosine inhibition of neuronal glutamate release. Nature *316:* 148–150.

Dolphin, A. C., S. R. Forda, and R. H. Scott (1986) Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. J. Physiol. (Lond.) *373:* 47–61.

Dunne, M. J., and O. H. Petersen (1986) GTP and GDP activation of K^+ channels that can be inhibited by ATP. Pfluegers Arch. *407:* 564–565.

Eckstein, F., D. Cassel, H. Levkovitz, M. Lowe, and Z. Selinger (1979) Guanosine 5′-*O*-(2-thiodiphosphate). An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. J. Biol. Chem. *254:* 9829–9834.

Gahwiler, B. H., and D. A. Brown (1985) $GABA_B$-receptor-activated K^+ current in voltage-clamped CA3 pyramidal cells in hippocampal cultures. Proc. Natl. Acad. Sci. USA *82:* 1558–1562.

Gilman, A. G. (1984) G-proteins and dual control of adenylate cyclase. Cell *36:* 577–579.

Greene, R. W., and H. L. Haas (1985) Adenosine action on CA1 pyramidal neurones in rat hippocampal slices. J. Physiol. (Lond.) *366:* 119–127.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. Sigworth (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pfluegers Arch. *391:* 85–100.

Henderson, J. F., and A. R. P. Paterson (1973) *Nucleotide Metabolism,* Academic, New York, London.

Holz, G. G., S. G. Rane, and K. Dunlap (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. Nature *319:* 670–672.

Hudson, T. H., and J. N. Fain (1983) Forskolin activated adenylate cyclase. Inhibition by guanylyl-5′yl imidodiphosphate. J. Biol. Chem. *258:* 9755–9761.

Kakei, M., A. Noma, and T. Shibasaki (1985) Properties of adenosine-triphosphate-regulated potassium channels in guinea-pig ventricular cells. J. Physiol. *363:* 441–462.

Kostyuk, P. G., O. A. Krishtal, and V. I. Pidoplichko (1975) Effect of internal fluoride and phosphate on membrane currents during intracellular dialysis of nerve cells. Nature *257:* 691–693.

Kostyuk, P. G., O. A. Krishtal, and V. I. Pidoplichko (1981) Calcium inward current and related charge movements in the membrane of snail neurons. J. Physiol. (Lond.) *310:* 403–421.

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.

Lotan, I., N. Dascal, Y. Oron, S. Cohen, and Y. Lass (1985) Adenosine-induced K^+ current in *Xenopus* oocyte and the role of adenosine 3′,5′-monophosphate, Mol. Pharmacol. *28:* 170–177.

MacDonald, R. L., J. H. Skerritt, and M. A. Werz (1986) Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. J. Physiol. (Lond.) *370:* 75–90.

Madison, D. V., and R. A. Nicoll (1986) Cyclic adenosine 3′,5′-monophosphate mediates β-receptor actions of noradrenaline in rat hippocampal pyramidal cells. J. Physiol. (Lond.) *372:* 245–259.

Nestler, E. J., and P. Greengard (1984) *Protein Phosphorylation in the Nervous System,* Wiley, New York, p. 20.

Newberry, N. R., and R. A. Nicoll (1985) Comparison of the action of baclofen with γ-aminobutyric acid on rat hippocampal pyramidal cells *in vitro*. J. Physiol. (Lond.) *360:* 161–185.

North, R. A., and J. T. Williams (1985) On the potassium conductance increased by opioids in rat locus coeruleus neurons. J. Physiol. (Lond.) *364:* 265–280.

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 potassium channel. Nature *317:* 536–538.

Phillis, J. W., and P. H. Wu (1981) The role of adenosine and its nucleotides in central synaptic transmission. Prog. Neurobiol. *16:* 187–239.

Premont, J., M.-C. Daguet-de Montety, A. Herbet, J. Glowinski, J. Bockaert, and A. Prochiantz (1983) Biogenic amine- and adenosine-sensitive adenylate cyclases in primary cultures of striatal neurons. Dev. Brain Res. *9:* 53–61.

Roof, D., M. Applebury, and P. C. Sternweis (1985) Relationships within the family of GTP-binding proteins isolated from bovine central nervous system. J. Biol. Chem. *260:* 16242–16249.

Seamon, K. B., W. Padgett, and J. W. Daly (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membrane and intact cells. Proc. Natl. Acad. Sci. USA *78:* 3363–3367.

Segal, M. (1982) Intracellular analysis of postsynaptic actions of adenosine in the rat hippocampus. Eur. J. Pharmacol. *79:* 193–199.

Snyder, S. H. (1985) Adenosine as a neuromodulator. Annu. Rev. Neurosci. *8:* 103–124.

Sternweis, P. C., and J. D. Robishaw (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. *259:* 13806–13813.

Trussell, L. O., and M. B. Jackson (1985) Adenosine-activated potassium conductance in cultured striatal neurons. Proc. Natl. Acad. Sci. USA *82:* 4857–4861.

Trussell, L. O., and M. B. Jackson (1986) A GTP-binding protein mediates an adenosine-activated K^+ current independently of cAMP. Soc. Neurosci. Abstr. *12:* 15.

Tsien, R. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: Design, synthesis, and properties of prototypic structures. Biochemistry *19:* 2396–2404.

Ueda, T., S.-H. Chueh, M. W. Noel, and D. L. Gill (1986) Influence of inositol 1,4,5-trisphosphate and guanine nucleotides on intracellular calcium release within the N1E-115 neuronal cell line. J. Biol. Chem. *261:* 3184–3192.

Yakel, J. L., L. O. Trussell, and M. B. Jackson (1986) Serotonergic responses in cultured mouse striatal neurons. Soc. Neurosci. Abstr. *12:* 726.