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22. I calculated the *L. nigrescens* biomass (b), wet weight, for three types of plants according to the following formulas: (i) for undamaged plants, $b = 0.7208\ d$ (where d is the maximum diameter of holdfast); (ii) for half-damaged plants (less than 50% of the stipes dead), $b = 0.5593\ d$; and (iii) for fully damaged plants (more than 50% of the stipes dead), $b = 0.2035\ d$.
23. *Lessonia nigrescens* biomass determinations carried out on six different opportunities (1982 to 1984) at Las Cruces showed values ranging between 13 to 20 kg m^{-2}, wet weight, at the upper intertidal border and 27 to 38 kg m^{-2} at the middle and lower borders. The mean overall biomass of this kelp at Los Molles, central Chile, was about 33 kg m^{-2} [B. Santelices, "Manejo de praderas de *Lessonia nigrescens* en Chile Central" (final report, Subsecretaria de Pesca, Chile, 1981)].
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Activation of Muscarinic Potassium Currents by ATPγS in Atrial Cells

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Intracellular perfusion of atrial myocytes with adenosine 5′-(γ-thio) triphosphate (ATPγS), an ATP analog, elicits a progressive increase of the muscarinic potassium channel current, $I_{K(M)}$, in the absence of agonists. In this respect, ATPγS mimics the actions of guanosine triphosphate (GTP) analogs, which produce direct, persistent activation of the guanyl nucleotide–binding (G) protein controlling the $K^+_{(M)}$ channel. The effect of ATPγS on $I_{K(M)}$, however, differs from that produced by GTP analogs in two aspects: it requires relatively large ATPγS concentrations, and it appears after a considerable delay, suggesting a rate-limiting step not present in similar experiments performed with guanosine 5′-(γ-thio) triphosphate (GTPγS). Incubation of atrial homogenates with [^{35}S]ATPγS leads to formation of significant amounts of [^{35}S]GTPγS, suggesting that activation of $I_{K(M)}$ by ATPγS arises indirectly through its conversion into GTPγS by cellular enzymes. ATPγS is often used to demonstrate the involvement of protein phosphorylation in the control of various cellular processes. The finding that cytosolic application of ATPγS can also lead to G-protein activation implies that experiments with ATPγS must be interpreted with caution.

INTERNAL DIALYSIS OF ATRIAL MYOcytes with hydrolysis-resistant GTP analogs evokes muscarinic receptor–independent activation of $I_{K(M)}$ (*1, 2*) with an order of effectiveness (GTPγS > GMP-PNP ≃ GTP > GMP-PCP; where GMP-PNP is guanylyl imidodiphosphate and GMP-PCP is guanylyl (β,γ-methylene)-diphosphonate) that parallels their binding affinities to purified G proteins (*3, 4*). We found (*5*) that intracellular application of ATPγS, an ATP analog, has effects that closely resemble those of GTP analogs (*1, 6*) on $I_{K(M)}$. This result could be interpreted as a lack of specificity of the nucleotide site involved in muscarinic activation of cardiac K^+ channels, thus weakening the G-protein transduction hypothesis, since ATP interacts poorly, or not at all, with the GTP binding site of purified G proteins (*7*). Alternatively, the actions of ATPγS could stem from (i) contamination by GTPγS, (ii) formation of a stable thiophosphorylated protein involved in channel gating, or (iii) conversion of ATPγS into its guanosine counterpart. The experiments reported here were designed to distinguish among these possibilities.

The effects of intracellular application of ATPγS on K^+ currents were examined in single myocytes dissociated enzymatically from bullfrog atrium, with the tight-seal whole-cell voltage clamp technique (*1, 8*). Calcium and Na^+ currents were blocked by extracellular cadmium and tetrodotoxin, respectively. Outward and inward currents were measured at the end of 250-ms pulses from a holding potential of −85 to −45 mV and −135 mV, respectively. Figure 1A shows these values, plotted as a function of time, beginning 1 min after the disruption of the patch membrane; the patch pipette solution contained 2.5 m*M* ATPγS. A gradual increase in both inward and outward current is observed after 7 min of dialysis. Superfusion of the cell with 1 μ*M* acetylcholine (ACh) 10 min after patch rupture causes a rapid, albeit small, increase in current; washout of agonist 1 min later has little effect on the current levels, indicating persistent activation of $I_{K(M)}$. The membrane currents elicited by the test pulses are shown in Fig. 1B, which illustrates the appearance of the characteristic relaxation of $I_{K(M)}$ (*1, 9*) well before agonist was applied. The current-voltage (*I-V*) relations measured at various times during this experiment are plotted in Fig. 1C. It is clear that the voltage dependence of the current activated by ATPγS differs from that of the background K^+ channel, I_{K1}, and parallels that observed during ACh application. External application of 0.1 m*M* $BaCl_2$, but not 1 μ*M* atropine, completely blocks the membrane currents elicited by ATPγS. By these criteria, the current that develops during intracellular perfusion with ATPγS is indistinguishable from $I_{K(M)}$, whether elicited by agonist application or by intracellular dialysis with GTP analogs (*1*).

We examined the effect of loading cells with different ATPγS concentrations in experiments similar to that in Fig. 1. After receptor-independent activation had reached a steady level, 1 μ*M* ACh was added

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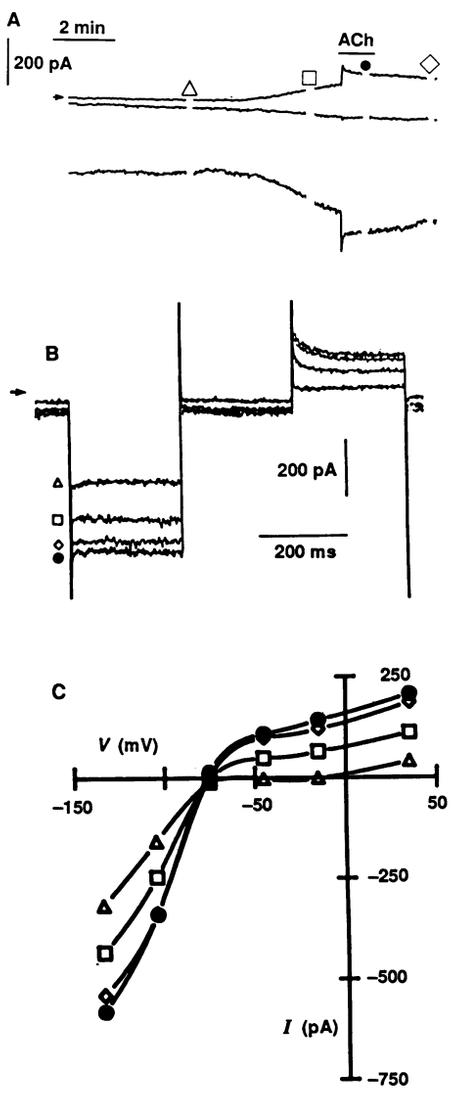
for a period of 1 min; the ACh response of each cell was then used to normalize the maximal rate of increase in the outward current. The maximal rate of spontaneous development of $I_{K(M)}$ in the presence of ATPγS increased from an unmeasurably small value at 0.1 m*M* (*10*) to a limiting value of about 0.17 per minute for the 0.5 to 2.5 m*M* concentration range (Table 1). In contrast, the rate of activation of $I_{K(M)}$ by GTPγS in the presence of a constant GTP concentration depends markedly on analog concentration and reaches a maximum of 0.3 per minute at saturating concentrations of analog (*1*). This difference in the maximal rates of activation suggests that the effects of ATPγS and GTPγS are limited by different processes. For GTP analogs, the limit reflects the rate at which guanosine diphosphate (GDP) dissociates from the G protein in the absence of agonist (*1*). The significantly lower maximal rate of agonist-independent activation of $I_{K(M)}$ observed with ATPγS implies the presence of an earlier and slower rate-limiting step. This hypothesis is further supported by the marked difference between GTPγS and ATPγS with respect to the delay observed between patch rupture and the onset of spontaneous activation: 0.1 m*M* GTPγS has observable effects within 2 min of cell perfusion [see also (*1, 2*)], whereas the increases in current produced by 0.5 to 2.5 m*M* ATPγS take 4 to 8 min to appear. Note that intracellular application of 0.1 m*M* GTPγS results in rates of receptor-independent activation that are similar to those observed with 0.5 to 2.5 m*M* ATPγS (Table 1).

Fig. 1. Activation of $I_{K(M)}$ by ATPγS. K^+ currents were measured in cells superfused with Ringer solution (90 m*M* NaCl, 20 m*M* Hepes, 5 m*M* $MgCl_2$, 2.5 m*M* KCl, 2.5 m*M* $CaCl_2$, 0.5 m*M* $CdCl_2$, 0.005 m*M* tetrodotoxin, *p*H 7.4 with NaOH). The internal solution contained 80 m*M* potassium aspartate, 30 m*M* KCl, 5 m*M* Hepes, 2.5 m*M* ATPγS, 2.5 m*M* MgCl, 1 m*M* EGTA, *p*H 7.4 with KOH (in all experiments Mg^{2+} and nucleotides were added in equimolar concentrations). (**A**) Recording of membrane currents at end of 250-ms pulses to −45 mV (upper trace) and −135 mV (lower trace) from a holding potential of −85 mV (middle trace). Currents in the last 25 ms of each pulse were averaged and plotted as a function of time. Blank spaces represent times at which *I-V* relations were obtained, at t = 5 (△), 9 (□), 10.75 (●), and 13 (◇) min. Arrow indicates zero current value. Traces begin 1 min after patch rupture; negative deflections represent inward currents. ACh (1 μ*M*) was applied at 10 min for a period of 1 min. (**B**) Current traces elicited by pulses to −45 and −135 mV from −85 mV at the times indicated by the corresponding symbols in (A). (**C**) *I-V* curves taken at times indicated by symbols in (A). Shown are averages of two to three current measurements at the end of each 250-ms voltage pulse.

The data in Table 1 provide indirect evidence that activation of $I_{K(M)}$ cannot be ascribed to contamination of ATPγS with GTPγS, because, if so, the rate of spontaneous activation would vary with the nominal ATPγS concentration. Moreover, thin-layer chromatography (TLC) of the four preparations of ATPγS used in these experiments reveals adenosine diphosphate (ADP) as the major impurity; there is no discernible contamination with GTPγS. In control experiments, we performed TLC analysis of solutions with various ATPγS/GTPγS ratios, thereby establishing a lower detection limit for GTPγS of 1 to 2%. However, we estimate that the response observed with ATPγS could only result from levels of contaminating GTPγS above 2.5% (Table 1).

When cells were loaded with another ATP analog, adenylyl imidodiphosphate (AMP-PNP, 2 to 5 m*M*), the membrane currents remained unaltered for up to 15 min (n = 8); exposure to 1 μ*M* ACh produced the usual response, which was fully reversible (Fig. 2). This result agrees with the observation that AMP-PNP does not affect single $K^+_{(M)}$ channels in membrane patches excised from atrial cells (*11*). The same pattern was observed in cells dialyzed with 2.5 m*M* adenylyl (β,γ-methylene)-diphosphonate (AMP-PCP; n = 3). These results indicate that the actions of ATPγS are not due to lack of specificity of the nucleotide site involved in $I_{K(M)}$ activation, which would result in spontaneous or at least irreversible activation of $I_{K(M)}$ by all three ATP analogs, as is observed with their guanosine counterparts (*1*). Furthermore, the lack of effect of AMP-PNP and AMP-PCP, which, in contrast with ATP or ATPγS, are not substrates for kinases or other enzymes that cleave the β-γ pyrophosphate bond (*12*), suggests that participation of ATPγS in a reaction involving transfer of the phosphate group underlies its action on $I_{K(M)}$. This reaction is probably not the thiophosphorylation of the channel or a closely asso-



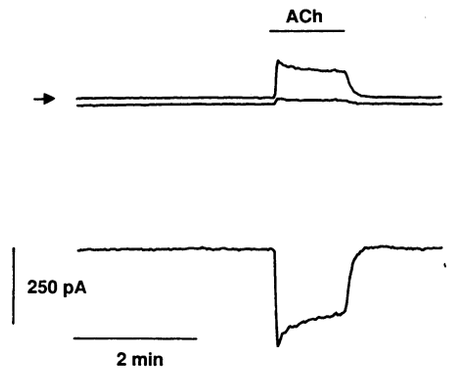


Fig. 2. $I_{K(M)}$ in the presence of AMP-PNP. Continuous recording of K^+ currents, beginning 7 min after patch disruption, in a cell perfused with 2 m*M* AMP-PNP. Other conditions are as in Fig. 1A. ACh (1 μ*M*) was applied at 10 min for a period of 1 min. Arrow indicates zero current value.

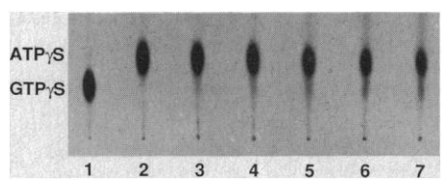


Fig. 3. Formation of [^{35}S]GTPγS by atrial homogenates in the presence of [^{35}S]ATPγS. The reaction mixture (50 μl) contained 5 μg of atrial homogenate [prepared as in (*16*)], 0.2 m*M* Mg · ATP, 50 μ*M* GTP, 0.5 m*M* [^{35}S]ATPγS (Du Pont Biotechnology Systems), 0.5 m*M* $MgCl_2$ in the standard intracellular solution described in Fig. 1. Incubation was for 25 min at 22°C. Aliquots (1 μl) were taken at 0.5, 5, 10, 15, 20, and 25 min (lanes 2 to 7), and processed as in (*17*). Lane 1, [^{35}S]GTPγS (Du Pont Biotechnology Systems), diluted and applied directly to the plate. Similar results were obtained in 15 such experiments.

Table 1. Agonist-independent activation of $I_{K(M)}$ by ATPγS and GTPγS. Conditions were as in Fig. 1. Rates were calculated by using the slope of the rise of outward current and are expressed as fraction (mean ± SEM) of the steady-state $I_{K(M)}$ outward current (steady-state currents are defined as current measured 55 s after ACh application). ACh (1 μ*M*) was added for a period of 1 min either 10 or 15 min after patch disruption.

Nucleotide	Concentration (m*M*)	Rate of $I_{K(M)}$ activation (min^{-1})	Delay (min)
ATPγS	0.1 (9)*	0†	–†
	0.5 (3)	0.17 ± 0.05	7.2 ± 0.9
	2.5 (3)	0.18 ± 0.06	6.4 ± 2.1
GTPγS	0.1 (4)	0.21 ± 0.04	1.3 ± 0.5

*Number of cells is shown in parentheses. †At 0.1 m*M*, ATPγS did not change cell currents in the absence of agonist in experiments lasting up to 18 min [but see (*10*)].

ciated protein because normal ACh responses were observed (Fig. 2) after prolonged dialysis with AMP-PNP, a competitive inhibitor of protein kinases (*13*). In this respect, stimulation of $I_{K(M)}$ by ACh differs from the activation of cardiac Ca^{2+} channels by β-adrenergic agonists, which is known to depend on protein phosphorylation and is markedly reduced by intracellular application of AMP-PNP (*14*). Moreover, although Ca^{2+} channels are stimulated by intracellular ATPγS in the absence of agonists, increases of I_{Ca} are evident shortly after perfusion with the analog is begun (*15*), without the long delay observed here.

We next tested the hypothesis that activation of $I_{K(M)}$ by ATPγS results from its conversion into GTPγS by cellular enzymes. Bullfrog atria were homogenized (*16*) and then incubated with [^{35}S]ATPγS for 25 min; during this period aliquots of the reaction mixture were taken and analyzed by TLC, followed by autoradiography of the plates (*17*). Figure 3 shows the autoradiogram of an experiment performed with 0.5 m*M* [^{35}S]ATPγS; clearly bullfrog atria contain enzymes that can metabolize ATPγS. The most prominent spot resulting from these reactions can be identified as [^{35}S]GTPγS (compare mobility with that of the [^{35}S]GTPγS standard, lane 1). Formation of the GTP analog is barely detectable during the first 5 min of reaction; after 25 min a significant fraction of the label originally present in ATPγS appears in the GTPγS spot. The slow time course of GTPγS formation is not unexpected and agrees with reports that utilization of thiophosphate by phosphotransferases occurs at rates much lower than those observed with phosphate (*18*). Under these conditions, a delay in appearance of G protein–related effects is expected, since GTPγS has to accumulate in order to effectively compete with cellular GTP for the activating site in the G protein (*1*). Taken together, these observations imply that the slow process that underlies the latency of ATPγS-induced $I_{K(M)}$ is the conversion of ATPγS into GTPγS. Note that the presence of this saturable conversion process between the introduction of ATPγS into the cell and GTPγS-dependent G-protein activation is likely to set a limit on the maximal rate of agonist-independent activation of $I_{K(M)}$, which can be slower than the rate-limiting step measured in cells perfused with GTP analogs. If so, the rates shown in Table 1 might actually reflect the maximal velocity for GTPγS formation, suggesting that saturation is already achieved at an ATPγS concentration of 0.5 m*M*. Although at this point our results do not allow us to identify the enzyme (or enzymes) responsible for the formation of GTPγS from ATPγS, the most likely candidate is nucleosidediphosphate kinase (NDPK; EC 2.7.4.6), which catalyzes the reaction ATP + NDP → ADP + NTP and has been shown to utilize nucleoside thiotriphosphates such as ATPγS (*18*). ATP is the preferred substrate for the cytosolic form of the beef heart enzyme, whereas GTP is a poor phosphate donor (*19*). We observed a similar specificity for the adenosine moiety of the nucleoside triphosphate in frog atrial homogenates, which do not convert [^{35}S]GTPγS into [^{35}S]ATPγS in experiments similar to that shown in Fig. 3.

Our data are consistent with a central role for transphosphorylation in the activation of $I_{K(M)}$ by ATPγS. The presence of phosphotransferases in this preparation accounts for the requirement for extensive metabolic intervention in order to deplete GTP in frog atrial cells (*1*). In fact, maintenance of GTP levels at the expense of ATP is probably a necessary condition for G-protein activity during periods of increased GTP turnover brought about by maximal receptor stimulation. This ability to "clamp" the cellular GTP concentration at levels compatible with optimal cell function is observed not only in amphibian atrial cells but also in cultured mammalian neurons (*20*). Preliminary experiments show that highly purified sarcolemmal preparations obtained from whole bullfrog heart (*21*) can convert ATPγS into GTPγS even more efficiently than atrial homogenates, indicating that part of the phosphotransferase activity is located in the plasma membrane. This finding suggests that GTP can be generated in the immediate vicinity of membrane-bound G proteins and is consistent with reports of interaction between G proteins and NDPK (*22*).

Finally, in view of the ubiquity of transphosphorylating enzymes such as NDPK and adenylate (and guanylate) kinase, and because not only ATPγS but also a variety of thiophosphate nucleotide analogs (ADPβS, GDPβS, and AMPS (*23*)] are substrates for these enzymes, results obtained when thiophosphate compounds are introduced in intact cells must be interpreted with great caution, and any conclusions concerning the origin of these effects should be supported by independent criteria (*15*).

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16. Atria were dissected from bullfrog (*Rana catesbeiana*) hearts. The tissue (0.1 g wet weight) was disrupted by scraping with glass slides, suspended in 250 μl of standard intracellular solution (see legend to Fig. 1) and homogenized in a Potter-Elvehjem (0.5-ml capacity) homogenizer.
17. Chromatography was run on polyethyleneimine cellulose-coated acetate sheets (Sigma) in 0.5*M* NaCl, 0.75*M* tris-HCl, *p*H 7.5 for 4 hours (ascending, continuous development). Each aliquot was applied to sheets spotted with 2 m*M* each ATPγS and GTPγS, as ultraviolet (UV) visualization markers and carriers. Chromatograms were exposed to Kodak XO-MAT AR film for 30 to 90 min.
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