

A G_s Protein Couples P_2 -purinergic Stimulation to Cardiac Ca Channels without Cyclic AMP Production

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ABSTRACT P_2 -purinergic stimulation of the L-type Ca current induced by the external application of 100 μ M ATP γ S was investigated in rat ventricular cardiomyocytes using the whole-cell patch-clamp technique. The purinergic-induced increase in I_{Ca} was slow and monophasic and reached a steady state within 3 min. In contrast to β -adrenergic stimulation, after a brief agonist application the current did not continue to increase on washout; recovery started immediately after agonist removal. The P_2 -purinergic increase in I_{Ca} was significantly less in the presence of GDP β S, but it occurred much faster and was twice as large when a low dose of GTP γ S (100 μ M) was added to a GTP-containing internal medium. This suggests that the I_{Ca} increase was mediated by a G protein. Based on electrophoretic mobility and susceptibility to cholera toxin and anti- $G\alpha_s$ serum, it is proposed that the G protein involved during purinergic-induced I_{Ca} stimulation is an isoform of G_s , not coupled to the adenylyl cyclase, since the cyclic AMP level was unaffected. High intracellular GTP γ S (1 mM) maximally activated I_{Ca} so that neither β -adrenergic nor P_2 -purinergic agonists further increased I_{Ca} . In the absence of GTP and an ATP-regenerating system, GTP γ S was much more potent in increasing basal I_{Ca} and supporting purinergic stimulation. This indicates that a nucleoside diphosphate kinase activity might replenish endogenous GTP; GTP exchange with GTP γ S on the G protein was promoted by the P_2 -purinergic stimulation and led to a reversible and reproducible increase in I_{Ca} . In the presence of 3 mM internal ATP γ S, the P_2 -purinergic stimulation was also reversible and reproducible. Moreover, under these conditions (ATP γ S or GTP γ S) the increase in I_{Ca} was not maintained during prolonged agonist application. Such an inhibition occurred slowly and irreversibly; it might be related to the threefold increase in cyclic GMP. In conclusion, we propose that extracellular ATP induces both a stimulatory and an inhibitory effect on I_{Ca} , probably mediated by subtypes of P_2 -purinergic receptors. An isoform of the G_s protein is likely to mediate the stimulation.

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INTRODUCTION

In heart cells the Ca current, I_{Ca} , which plays an essential role in controlling the electrical activity and contractility, is modulated by hormones and neurotransmitters (see Hartzell, 1988). The β -adrenergic-induced increase in I_{Ca} has been extensively studied. The increase in I_{Ca} follows the stimulation of adenylyl cyclase; the resulting increase in cyclic AMP activates a cyclic AMP-dependent protein kinase (PKA) which phosphorylates the Ca channel proteins or a nearby protein (Trautwein and Hescheler, 1990). Stimulation of the cyclase after binding of agonist to the receptor implies interaction of the receptor with a stimulatory guanine nucleotide regulatory protein (G_s) localized at the inner face of the plasma membrane (Brown and Birnbaumer, 1990). Initially, a direct interaction of adenylyl cyclase with the G protein α subunit was shown in reconstitution experiments (Feder, Im, Klein, Hekman, Holzhöfer, Dees, Levitzki, Helmreich, and Pfeuffer, 1986). More recently it was shown that the $\beta\gamma$ subunits also either activate or inhibit the different subtypes of adenylyl cyclase (Tang and Gilman, 1991). A close control of a channel by a G protein was initially reported for K channels in cardiac cells (Kurachi, Nakajima, and Sugimoto, 1986). With regard to the Ca channel, a direct coupling of a G protein to dihydropyridine (DHP) binding sites is supported by the facts that guanylyl-imidodiphosphate (GMP-PNP) increases the ability of a DHP agonist to displace a DHP antagonist (Bergamaschi, Govoni, Cominetti, Parenti, and Trabucchi, 1988) and that guanosine 5'-O-3-thiotriphosphate (GTP γ S) enhances charge movement and I_{Ca} while phosphorylation by PKA increases only I_{Ca} (Garcia, Gamboa-Aldeco, and Stefani, 1990). There is also evidence that $G_s\alpha$ comigrates with the DHP receptor in the skeletal muscle (Hamilton, Codina, Hawkes, Yatani, Sawada, Strickland, Froehner, Spiegel, Toro, Stefani, Birnbaumer, and Brown, 1991). A direct gating of the Ca channel by G_s proteins, independent of protein kinase phosphorylation, has been suggested by applying the preactivated α_s subunit to the cytoplasmic side of isolated membrane patches or to Ca channels incorporated into planar lipid bilayers (Yatani, Codina, Imoto, Reeves, Birnbaumer, and Brown, 1987; Imoto, Yatani, Reeves, Codina, Birnbaumer, and Brown, 1988; Yatani, Imoto, Codina, Hamilton, Brown, and Birnbaumer, 1988). Moreover, it has recently been suggested that cyclic AMP-dependent phosphorylation could not explain all the effects of β -adrenergic agonists on I_{Ca} and that a parallel "membrane-delimited" pathway was also involved in this stimulation (Yatani and Brown, 1989; Shuba, Hesslinger, Trautwein, McDonald, and Pelzer, 1990; Cavalié, Allen, and Trautwein, 1991; but see Hartzell, Méry, Fischmeister, and Szabo, 1991).

Purinergic receptors control many cellular functions; they have been classified into two main categories (Burnstock, 1981). The P_1 purinoceptors most sensitive to adenosine are linked to adenylyl cyclase in either an inhibitory (A_1) or excitatory (A_2) manner. The P_2 purinoceptors for which ATP is the preferred agonist induce prostaglandin synthesis (Needleman, Minkes, and Douglas, 1974; Schwartzman, Pinkas, and Raz, 1981; Takikawa, Kurachi, Mashima, and Sugimoto, 1990) and increase inositol-lipid metabolism (Legssyer, Poggioli, Renard, and Vassort, 1988; see Olsson and Pearson, 1990 for a review on the cardiovascular system). More specifically, in cardiac tissues and particularly in the auricles P_1 -purinergic stimula-

tion leads to both negative chronotropic and inotropic effects which could be mediated by an increase in K current (Belardinelli and Isenberg, 1983) and a decrease in Ca current (Cerbai, Klöckner, and Isenberg, 1988). Adenosine, through A_2 receptors, was also shown to activate the adenylyl cyclase in the ventricle and a positive inotropy was reported in the chick (Xu, Kong, and Liang, 1992) but not observed in the guinea pig (Behnke, Müller, Neumann, Schmitz, Scholz, and Stein, 1990). On the other hand, extracellular ATP in the ventricles or in pertussis toxin-treated auricles induced a positive inotropy (Legssyer et al., 1988; Scamps, Legssyer, Mayoux, and Vassort, 1990a); this was attributed to P_2 -purinergic stimulation since ATP was much more efficient in increasing the Ca current than adenosine in both frog and rat ventricular cells (Alvarez, Mongo, Scamps, and Vassort, 1990; Scamps et al., 1990a). The increase of I_{Ca} under P_2 stimulation was pertussis toxin insensitive. Recently, a P_3 -type of purinoceptor was defined on the basis that it is activated only by triphosphate adenosine derivatives and that it has an absolute requirement of Mg ions while the P_2 -purinergic increase in I_{Ca} does not. P_3 purinostimulation leads to cell depolarization and possibly arrhythmias as a consequence of a transient acidosis due to activation of the Cl/HCO₃ exchanger (Scamps and Vassort, 1990; Pucéat, Clément, Scamps, and Vassort, 1991a; Pucéat, Clément, and Vassort, 1991b).

This work investigates in more detail the possible pathways involved in the rise of I_{Ca} under P_2 purinoceptor stimulation in rat cardiac cells. A direct activation of the Ca conductance, mediated by a G_s protein without variations in cyclic AMP level, is proposed to account for the ATP-induced increase in I_{Ca} . Furthermore, during prolonged agonist application in the presence of hydrolyzable-resistant analogues of GTP γ S or adenosine 5'-O-3-thiotriphosphate (ATP γ S), an inhibition of I_{Ca} was observed.

METHODS

Isolation of Single Cells

Single ventricular cells from male Wistar rats (180–250 g) were enzymatically dispersed as described previously (Pucéat, Clément, Lechêne, Pelosin, Ventura-Clapier, and Vassort, 1990) and kept at 37°C before the experiments. The yield of rod-shaped cells was generally >70%.

Solutions and Drugs

Control external solution contained: 117 mM NaCl, 20 mM CsCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, 5 mM D-glucose, and 50 μ M tetrodotoxin; pH was adjusted to 7.4 with NaOH. Patch electrodes (0.5–1 M Ω) were filled with a standard internal solution that contained (mM): 120 CsCl, 5 Cs₂EGTA, 6.8 MgCl₂, 5 Na₂PCr, 5 Na₂ATP, 0.4 Na₂GTP, 0.06 CaCl₂ (pCa 8.7), and 20 HEPES; pH was adjusted to 7.2 with CsOH. In a few experiments, a low Mg–low substrate internal solution was used (mM): 120 CsCl, 10 NaCl, 5 Cs₂EGTA, 0.06 CaCl₂, 1 MgATP, and 20 HEPES, pH 7.2.

The drugs used in these experiments were isoproterenol, neomycin sulfate, phorbol 12-myristate 13-acetate (PMA), indomethacin, nordihydroguaiaretic acid, cholera toxin, 5'-guanylylimidodiphosphate (GppNHp), guanosine 5'-O-2-thiodiphosphate (GDPBS), isobutylmethylxanthine (IBMX) (Sigma, L'Isle d'Abeau Chesnes, France); ATP γ S, GTP γ S (Boehringer Mannheim, Biochemica, France), and [³²P]NAD (Dupont de Nemours, Paris, France). GF

109203X was a gift from Glaxo (Les Ulis, France). The electrophysiological experiments were performed at room temperature (21–24°C). Incubations of cells with toxins or with the agonists for biochemical assays were done at 37°C.

Electrophysiological Recordings

I_{Ca} was recorded with the whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). In each experiment the cell was depolarized every 4 s from -70 mV holding potential to 0 mV for 200 ms with a patch-clamp amplifier (model RK-300; Biologic, Claix, France) and L-type I_{Ca} was measured on-line by a Compaq 286 Desk-Pro computer (COMPAQ Computer Corp., Houston, TX) as the difference between peak inward current and current at the end of the 200-ms depolarization. We have previously shown that in rat ventricular cells, T-type Ca current is not present (Scamps, Mayoux, Charlemagne, and Vassort, 1990b). In few experiments, a residual I_{Na} could be seen when performing $I-V$ curves. This residual Na current was inhibited by ATP or ATP γ S application and thus could not account for our observations (Scamps, F., B. Turan, and G. Vassort, manuscript in preparation). Currents were digitized at 10 kHz (12-bit A/D converter). Before I_{Ca} recording, membrane capacitance (C_m) was measured as previously described (Scamps et al., 1990b).

Biochemical Techniques

Cyclic AMP and cyclic GMP measurements. To measure cyclic AMP or cyclic GMP content of cardiac cells, batches of 4×10^5 cells/ml were incubated at 37°C for 5 min in the presence of 100 μ M IBMX. Then 100 μ M ATP γ S or 10 μ M isoproterenol was added for 5 or 3 min, respectively. The incubations were rapidly stopped by addition of trichloroacetic acid (TCA). After centrifugation, the concentration of cyclic nucleotides was determined in the supernatant after the extraction of TCA with water-saturated ether. A commercially available kit using a high specificity binding protein assay (TRK 432; Amersham International, Amersham, UK) was used to determine cyclic AMP. Cyclic GMP was measured by radioimmunoassay following a succinylation of the cyclic nucleotide according to Cailla, Vannier, and Delaage (1976).

Preparation of cardiomyocyte membranes. Cardiomyocyte membranes were prepared according to Murakami and Yasuda (1986). Cells were diluted with 3 ml of buffer A (50 mM NaH₂PO₄, pH 7.5, 5 mM EDTA, 5 mM MgCl₂) and homogenized with a potter. Homogenates were spun at 1,000 g for 10 min at 4°C. Supernatant fluids were then spun at 30,000 g for 1 h. Pellets (crude membranes) were suspended in buffer A containing 1 mM DTT and stored at -20°C .

ADP ribosylation with cholera toxin. ADP ribosylation with cholera toxin was done according to Ribeiro-Neto, Mattera, Grenet, Sekura, Birnbaumer, and Field (1987). Cholera toxin (1 mg/ml) was activated for 20 min at 31°C in the presence of 25 mM DTT. ADP ribosylation mixture (50 μ l) contained 300 mM NaH₂PO₄, pH 7.5, 2 mM EDTA, 11 mM MgCl₂, 0.4 mM GppNHp, 100 μ M NADP, 10 mM thymidine, 2.5 mM DTT, 10 μ M [³²P]NAD (10 μ Ci/assay), 100 μ g/ml activated cholera toxin, and crude membranes (50–100 μ g). Samples were incubated for 1 h at 31°C. 50 μ l of SDS-PAGE sample buffer was added to stop the reaction.

SDS-PAGE. 11% polyacrylamide gels were used. After SDS-PAGE, gels were stained with Coomassie R250, destained, dried, and autoradiographed for 3 d with intensifying screen. Regions of the gels that corresponded to 44-kD cholera toxin substrate bands of the autoradiograms were excised and the amount of radioactivity was quantified by the use of a β counter.

Immunoblot analysis. For immunoblot analysis, cardiomyocyte crude membranes were subjected to SDS-PAGE. The separated proteins were then transferred from the gel to a nitrocellulose sheet using an electroblotting apparatus operated at a constant current of 190

mA for 16 h. Subsequent procedures were modified according to Foster, McDermott, and Robishaw (1990). After the transfer of proteins, the nitrocellulose was incubated with buffer B (50 mM Tris-Cl, pH 8.0, 2 mM CaCl₂, 80 mM NaCl, 0.02% NaN₃, 5% nonfat dry milk, and 0.2% NP-40). The anti-G_{sa} antibody 584 was kindly provided by Dr. A. G. Gilman (University of Texas Health Science Center, Dallas, TX). It was diluted in buffer B (but 2% NP-40) and incubated with the blot for 1 h at 20°C. After three washes with buffer B, the blot was incubated with ¹²⁵I-labeled goat anti-rabbit IgG (3.5 × 10⁵ cpm/ml) for 1 h at 20°C, washed five more times with buffer B, and allowed to air dry. Autoradiography of the dried blot was performed. Regions of the blot that correspond to 44-kD G_s α subunit bands of the autoradiograms were excised and the amount of radioactivity was quantified by the use of a γ counter.

Results are expressed as mean ± SEM. Statistical analysis was done with a Student's *t* test. The differences were considered significant when *P* < 0.05.

RESULTS

Effect of P₂-purinergic Agonists on Characteristics of I_{Ca}

In a previous study we reported that ATP (1–30 μM) induced an increase in *I*_{Ca} amplitude associated with a leftward shift in the voltage dependence of the Ca channel (Scamps et al., 1990a). As illustrated in Fig. 1, ATPγS was also effective and

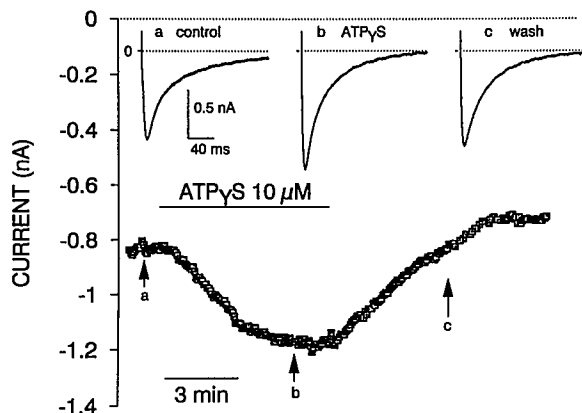


FIGURE 1. Effect of ATPγS on Ca current amplitude. *I*_{Ca} (□) was recorded every 4 s during 200-ms depolarizations to 0 mV (see Methods). (Inset) Ca current traces at indicated times (*C*_m = 110 pF).

increased *I*_{Ca} amplitude. The mean increase in peak *I*_{Ca} was 62.3 ± 4.4% (*n* = 6), 65.4 ± 3.7% (*n* = 4), and 54.6 ± 2.7% (*n* = 9) in the presence of 10 μM ATPγS, 100 μM ATPγS, or 10 μM ATP, respectively. The basal *I*_{Ca} density averaged 7.2 ± 0.4 pA/pF (*n* = 14). The increase in *I*_{Ca} amplitude induced by ATPγS was reversible and reproducible.

ATPγS increased the Ca current at every membrane depolarization; moreover, as did ATP, it induced a ~5-mV shift of the peak *I-V* curve toward hyperpolarized potentials (Fig. 2A). The availability curve of *I*_{Ca} was also shifted to more hyperpolarized potentials (*V*_f at 50% inactivation was -20.1 ± 1.3 and -26.8 ± 1.1 mV (*n* = 5) in control and in the presence of 10 μM ATPγS, respectively (*P* < 0.01).

A major difference between the actions of ATP and ATPγS was that ATPγS up to 100 μM did not significantly stimulate the P₃-purinergic receptors that lead to

acidosis and activation of a nonspecific cationic conductance (Scamps and Vassort, 1990; Pucéat et al., 1991b). For these reasons, and also due to the fact that ATP γ S is a slowly hydrolyzable analogue of ATP, we externally applied ATP γ S at 10 or 100 μ M in the following experiments.

Time Course of P₂-purinergic-induced Increase in Ca Current

During P₂-purinergic stimulation, the increase in I_{Ca} was slow and monophasic and required ~ 3 min to reach a steady state. The mean rate of increase, calculated as the slope of I_{Ca} increase, was 117 ± 21 pA \cdot min⁻¹ ($n = 8$) in the presence of 10 or 100 μ M

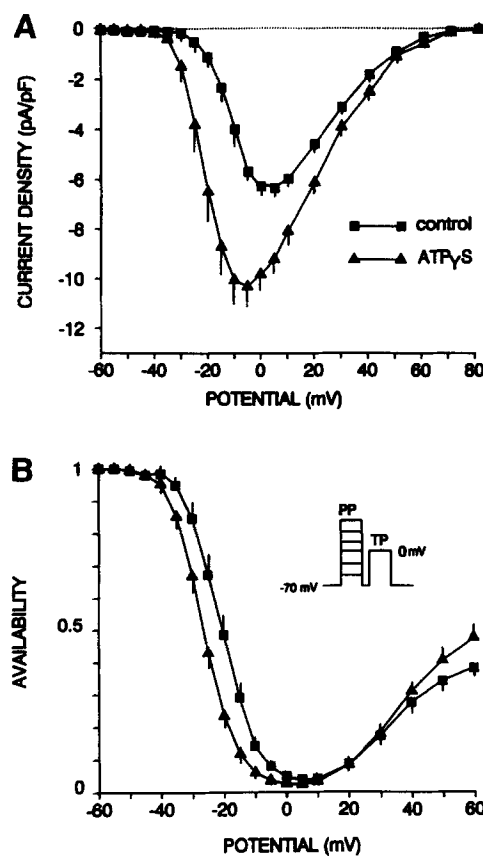


FIGURE 2. Effect of ATP γ S on Ca current characteristics. (A) Peak Ca current density-voltage relationships established in control (■) and in the presence of 10 μ M ATP γ S (▲), mean \pm SEM ($n = 4$). Densities instead of amplitudes are given to allow for comparison of I_{Ca} in different cells. The increase of I_{Ca} was significant when depolarizing pulses between -20 and $+10$ mV were applied. (B) Availability curves obtained in control (■) and in the presence of ATP γ S (▲), mean \pm SEM ($n = 4$). The leftward shift of the curve in the presence of ATP γ S was statistically significant (see text). The protocol used to construct $I-V$ and availability curves is shown in the inset to B. Prepulse potentials (PP; 200 ms, range -60 to $+80$ mV) were separated from the test pulse (TP; 0 mV, 200 ms) by a 3-ms return to -70 mV to better resolve I_{Ca} from the variable capacitive current. Pulse pairs were applied every 4 s. The currents elicited during the prepulse potentials were used to construct $I-V$ curves. The currents elicited during the test pulse were normalized to the current obtained without prepulse and plotted as a function of the prepulse potential. Curves are fitted by eye.

ATP γ S. In the experiment illustrated in Fig. 3, the effects of a short application of a P₂-purinergic and a β -adrenergic agonist were compared. The increase in current occurred only when the purinergic agonist was present in the external solution; I_{Ca} started to return toward its control value immediately after removal of the agonist. A second and sustained application of ATP γ S demonstrated the maximal effect of

ATP γ S on I_{Ca} . In contrast, when isoproterenol was applied for a brief period (< 10 s), the increase in I_{Ca} still developed after washing away the β -adrenergic agonist. The mean rate of increase under maximal stimulation with isoproterenol was 750 ± 70 pA \cdot min $^{-1}$ ($n = 5$).

Possible Metabolic Pathways

It was demonstrated in the rat heart that the P₂-purinergic stimulation is associated with the production of inositol triphosphate (InsP₃; Legssyer et al., 1988) due to activation of phospholipase C; a subsequent activation of the protein kinase C (PKC) by the simultaneously produced diacylglycerol might thus be expected. To investigate if PKC could play a role in the increase of I_{Ca} , we used a membrane-permeable phorbol ester, PMA, which directly activates this kinase. As shown in Fig. 4 A, the external application of 0.1 μ M PMA did not induce an increase in I_{Ca} amplitude, but rather decreased it as already reported by Tseng and Boyden (1991). The response to 10 μ M ATP γ S through the P₂-purinergic receptors was not affected by the

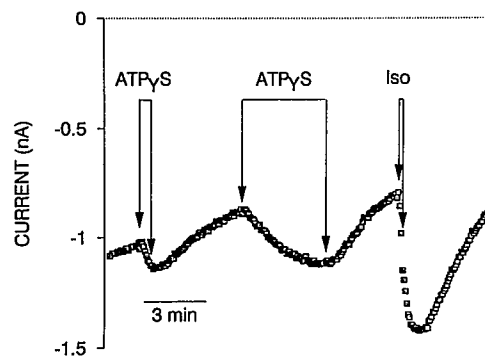


FIGURE 3. Kinetic aspects of P₂-purinergic and β -adrenergic stimulation of I_{Ca} . A brief (30 s) application of 100 μ M ATP γ S induced a measurable increase in I_{Ca} amplitude, which recovered on removal of the agonist. I_{Ca} was elicited by 200-ms depolarization to 0 mV. The second, more sustained application of ATP γ S allowed the full development of the ATP γ S effect. For comparison, a very brief application of isoproterenol elicited an increase in I_{Ca} which transiently continued developing after removal of the agonist.

presence of PMA (percent increase of I_{Ca} amplitude at 0 mV was $76.0 \pm 5.3\%$, $n = 3$). The prior incubation of cells for 20 min with 0.1 μ M PMA also did not affect the subsequent electrophysiological effects of ATP γ S (not shown).

To further support the lack of effect of PKC on the P₂-purinergic stimulation of I_{Ca} , a newly synthesized PKC inhibitor, GF 109203X, was used (Toullec, Pianetti, Coste, Bellevergue, Grand-Perret, Ajakane, Baudet, Boissin, Boursier, Loriolle, Duhamel, Charon, and Kirilovsky, 1991). Fig. 4 B shows that superfusion of 10 μ M GF 109203X induced a decrease in basal I_{Ca} ($38.1 \pm 4.4\%$, $n = 5$). After a steady state was reached, 10 μ M ATP γ S still induced a $55.2 \pm 11.5\%$ increase in I_{Ca} ($n = 5$), a value similar to that obtained in control experiments performed in the presence of the solvent (DMSO) ($53.9 \pm 13.2\%$ increase, $n = 2$).

In a previous study performed in frog heart, it was proposed that the increase in phosphatidylinositol turnover might in some way be involved in the I_{Ca} increase. In the presence of neomycin which, by binding to phosphoinositide biphosphate prevents its hydrolysis by the phospholipase C (Vergara, Tsien, and Delay, 1985), no

detectable increase in I_{Ca} could be recorded on applying ATP (Alvarez et al., 1990). Similar experiments were performed on rat heart cells in which a much larger increase in I_{Ca} is generally elicited by P_2 -purinergic stimulation. In the experiment illustrated in Fig. 4 C, neomycin was added at a concentration of 300 μ M together with 50 μ M cyclic AMP to the pipette solution to check cell dialysis. Soon after breaking the patch, I_{Ca} was very large, confirming diffusion of cyclic AMP, and

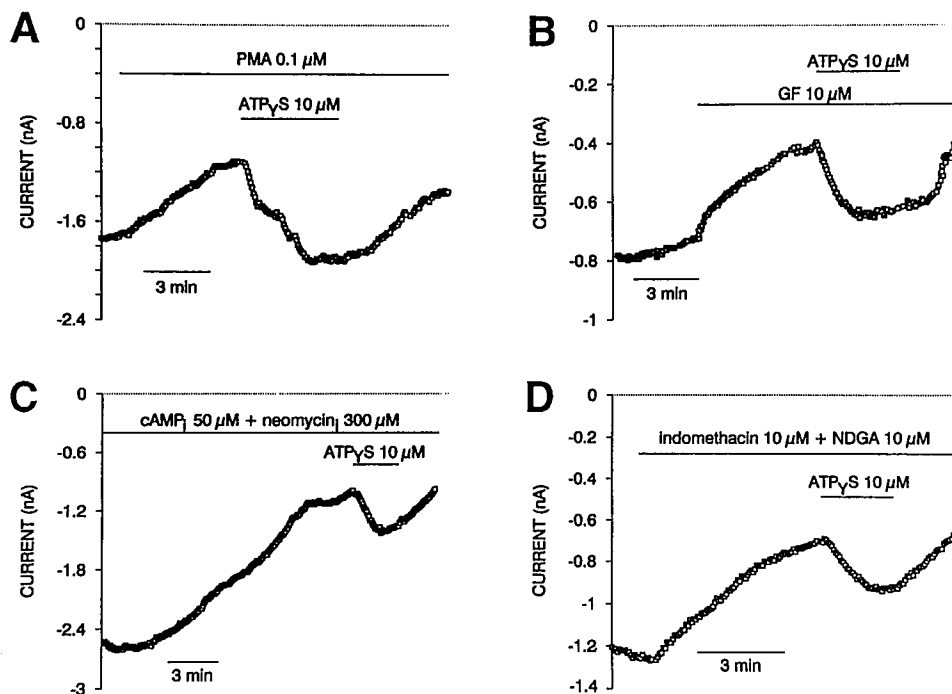


FIGURE 4. Evaluation of possible metabolic pathways involved in the P_2 -purinergic-induced increase in I_{Ca} . (A) External application of a PKC activator, PMA, induced a decrease in basal I_{Ca} amplitude but did not affect the P_2 -purinergic stimulation ($C_m = 225$ pF). (B) External application of a PKC inhibitor, GF 109203X, induced a decrease in basal I_{Ca} amplitude but did not affect the P_2 -purinergic stimulation ($C_m = 100$ pF). (C) Internal perfusion of the cell with neomycin, a phospholipase C inhibitor, induced a decrease in I_{Ca} amplitude but did not affect the P_2 -purinergic stimulation. Cyclic AMP was included in the patch electrode as a control for dialysis ($C_m = 160$ pF). (D) External application of indomethacin and NDGA, which are prostaglandin and leukotriene inhibitors, induced a decrease in I_{Ca} amplitude but did not prevent the P_2 -purinergic stimulation ($C_m = 155$ pF). In each experiment, I_{Ca} was elicited by 200-ms depolarization to 0 mV.

presumably neomycin, into the cell; I_{Ca} then decreased. This decrease was probably due to the nonspecific effects of neomycin as previously reported by Suarez-Kurtz and Reuben (1987). Nevertheless, the external application of 10 μ M ATP γ S still markedly enhanced I_{Ca} . Similar results were obtained in two other cells investigated under the same experimental conditions. In three other cells which were instead

incubated up to 90 min in the presence of 30 μM neomycin, ATP γS also induced an increase in I_{Ca} amplitude.

In many cell types including cardiomyocytes, the activation of the P_2 purinoceptors induces an increase in arachidonic acid leading to a synthesis of prostaglandins. Consequently, we investigated whether the products of arachidonic acid metabolism could account for the effects of P_2 -purinergic activation of I_{Ca} . Treatment of the cells for 5 or 10 min with 10 μM indomethacin, an inhibitor of the cyclooxygenase pathway, together with 10 μM nordihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathway, did not prevent the increase in I_{Ca} amplitude induced by the application of 10 μM ATP γS (Fig. 4D). During the course of this series of experiments the mean increase of I_{Ca} induced by ATP γS was $30.1 \pm 3.2\%$ as compared with $27.3 \pm 2.2\%$ ($n = 4$) for nontreated cells.

Involvement of a G Protein

To investigate whether the P_2 -purinergic-induced increase in I_{Ca} was mediated through a G protein, we used GDP βS , known to block G protein activation, and poorly hydrolyzable analogues of GTP, GppNHp, and GTP γS , known to induce sustained activation of the G proteins.

Effects of GDP βS . In preliminary experiments, a standard internal medium was used (see Methods), except that GTP was replaced with 1 mM GDP βS . Under this experimental condition, the P_2 -purinergic as well as the β -adrenergic stimulations were not affected at all. To allow GDP βS to better compete with endogenous GTP, a low Mg-low substrate internal solution was used (see Methods). When GDP βS was not added to this internal solution, 100 μM ATP γS and 1 μM isoproterenol induced 75.3 ± 12.5 and $78.1 \pm 7.8\%$ increases of I_{Ca} ($n = 4$), respectively (Fig. 5A). In the presence of 1 mM GDP βS (Fig. 5B) the increases in I_{Ca} under ATP γS and isoproterenol applications were $42.1 \pm 12.2\%$ ($n = 8$) and $32.1 \pm 2.6\%$ ($n = 5$), respectively, and were significantly different from control cells ($P < 0.05$ and $P < 0.005$, respectively).

Effects of GppNHp. Fig. 6 shows that internal perfusion with 1 mM GppNHp (standard internal medium without GTP) through the patch electrode apparently had no effect on basal I_{Ca} in this as well as in the four other cells tested. However, mean I_{Ca} density was significantly higher than under control conditions (10.1 ± 1.6 pA/pF, $n = 5$, compared with 7.2 ± 0.4 pA/pF, $n = 14$, $P < 0.05$). As stated in Methods, recordings of I_{Ca} were begun after membrane capacitance measurements, i.e., ~ 1 –2 min after patch breaking, so a slight stimulation might have already occurred. After a few minutes in this experimental condition, the application of 1 μM isoproterenol induced an increase in I_{Ca} amplitude that was poorly reversible on washout of the ligand (I_{Ca} increased by $40.1 \pm 8.5\%$, $n = 5$). The mean I_{Ca} density after isoproterenol was 14.4 ± 1.8 pA/pF, a value close to the one obtained with a standard internal medium (see Table I). The subsequent application of 100 μM ATP γS on top of the sustained isoproterenol effect induced a further significant increase in I_{Ca} amplitude ($18.8 \pm 6.2\%$, $n = 5$). The washout of ATP γS led to a pronounced decrease in I_{Ca} amplitude ($54.3 \pm 3.4\%$ decrease compared with maximal activated I_{Ca}). A second application of ATP γS was still able to increase I_{Ca} amplitude ($51.6 \pm 13.9\%$ increase relative to current amplitude just before ATP γS application, $n = 5$). This increase

induced by ATP γ S reapplication was not sustained but could be repeated. On the other hand, a second application of isoproterenol was without effect.

Effects of GTP γ S. Addition of 1 mM GTP γ S to the standard internal medium (in the absence of GTP) induced an increase in basal I_{Ca} which had reached its maximal steady level either at or just after the onset of I_{Ca} recording, presumably depending on cell dialysis (see Fig. 7A). In the presence of 1 mM GTP γ S, mean I_{Ca} density elicited by depolarization to 0 mV was 20.3 ± 2.8 pA/pF ($n = 9$), i.e., nearly a threefold increase relative to basal values (Table I). Under these steady-state

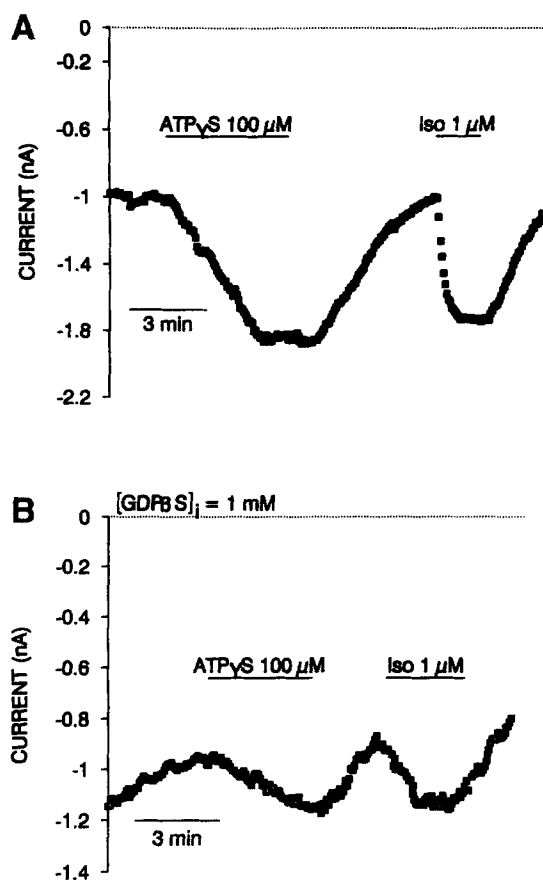


FIGURE 5. Effect of P₂-purinergic and β -adrenergic stimulations of I_{Ca} in the presence of GDP β S. (A) Control internal medium ($C_m = 170$ pF). (B) In the presence of 1 mM GDP β S ($C_m = 150$ pF).

conditions, application of 1 μ M isoproterenol had no effect on I_{Ca} amplitude, consistent with already maximal activation of the G_s protein by GTP γ S (not shown). Applications of 10 or 100 μ M ATP γ S also did not induce a further increase in I_{Ca} amplitude. However, while I_{Ca} retained its amplitude on washing out of the β -adrenergic agonist, a decrease of I_{Ca} amplitude was consistently observed on washout of 10 or 100 μ M ATP γ S in the six cells tested ($46.8 \pm 1.8\%$ decrease from the maximal GTP γ S-stimulated level) (Fig. 7A). In some other experiments, as illustrated in Fig. 7B, soon after the application of a high ATP γ S concentration (100

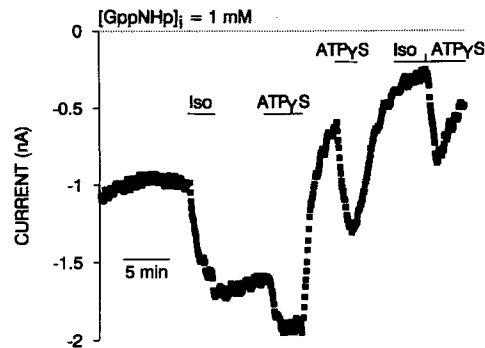


FIGURE 6. Effect of P_2 -purinergic and β -adrenergic stimulations of I_{Ca} in the presence of GppNHP, a hydrolysis-resistant analogue of GTP. I_{Ca} was elicited by 200-ms depolarization to 0 mV. Isoproterenol (*Iso*) and ATP γ S were applied as indicated at 1 and 100 μ M, respectively ($C_m = 135$ pF).

μ M) the GTP γ S-stimulated I_{Ca} decreased to a steady level and a further decrease was observed on washing out the agonist. A subsequent application of isoproterenol then had a weak positive effect on I_{Ca} (mean increase at 0 mV was $29.2 \pm 4.8\%$, $n = 5$) or no effect at all if it was not the first β -adrenergic stimulation of the cell. Forskolin at 10 μ M also did not resume the inhibitory effect. The inhibitory effect of ATP γ S was neither prevented nor reversed by the application of 100 μ M IBMX, a nonspecific phosphodiesterase inhibitor and a P_1 purinoceptor antagonist. That P_1 -purinergic stimulation was not involved in this inhibitory effect was reinforced by the lack of adenosine (100 μ M)-induced alterations of the GTP γ S-stimulated I_{Ca} during its application or on removal (not shown). A subsequent ATP γ S application was always able to transiently increase I_{Ca} .

In the presence of GTP γ S, ATP γ S still induced a shift in the availability curve (V_f at 50% inactivation was shifted from -26.6 to -34.6 mV and from -31 to -36.6 mV in two cells so investigated).

Effects of a low dose of GTP γ S in the presence of GTP. Addition of 100 μ M GTP γ S to the standard internal medium (in the presence of 400 μ M GTP) had only a weak or no effect on basal I_{Ca} amplitude. However, under this experimental condition, 100 μ M ATP γ S induced a pronounced increase in I_{Ca} amplitude which was twice the effect of ATP γ S in control conditions (Fig. 8A) ($125 \pm 3\%$ increase, $n = 3$ compared with $65.4 \pm 7.4\%$, $n = 4$ in standard internal solution, $P < 0.01$). The mean rate of increase was 269 ± 45 pA \cdot min $^{-1}$, a value significantly greater than the control value

TABLE I
Relative Increase in I_{Ca} Density under P_2 -purinergic Stimulation

	Control	Isoproterenol (1 μ M)	Cholera toxin (10 μ g/ml)	GTP γ S (1 mM)
I_{Ca} density (pA/pF)	7.2 ± 0.4	13.7 ± 0.6	$20.8 \pm 0.7^*$	$20.3 \pm 0.9^*$
ATP (% increase)	$54.6 \pm 2.7^{\ddagger}$	$40.7 \pm 3.1^{\S}$	2.6 ± 2.8	See text

I_{Ca} density is the value of I_{Ca} amplitude, measured at 0 mV, relative to the cell membrane capacitance, C_m (see Methods). Values are given as mean \pm SEM; number of experiments $9 \leq n \leq 14$. Statistical analysis was done with the Student's t test to compare I_{Ca} density under each experimental condition. * $P < 0.001$ relative to current densities either in control or in isoproterenol conditions. A paired test was used for the significance in the percentage increase in I_{Ca} density under external application of 10 μ M ATP or 100 μ M ATP γ S. $^{\ddagger}P < 0.001$; $^{\S}P < 0.01$.

($n = 3$, $P < 0.02$). As in the experiments performed with 1 mM internal GTP γ S or GppNHP (without added GTP), I_{Ca} decreased on washout of ATP γ S. Before a steady state was reached, the subsequent application of 1 μ M isoproterenol allowed I_{Ca} to recover its maximal amplitude. On isoproterenol removal, I_{Ca} stabilized at a rather high amplitude. Reapplication of ATP γ S, which did not significantly increase I_{Ca} above this apparent maximal level, induced a decrease in current and allowed control solution to further markedly reduce its amplitude. Subsequent ATP γ S applications were then able to transiently and reversibly increase I_{Ca} ; isoproterenol was ineffective.

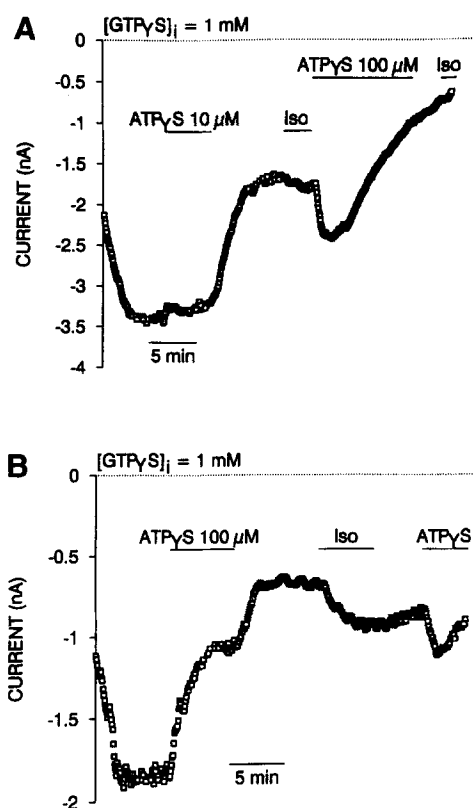


FIGURE 7. Effect of P_2 -purinergetic and β -adrenergic stimulations of I_{Ca} in the presence of GTP γ S, a hydrolysis-resistant analogue of GTP. (A) Effect of 10 μ M ATP γ S and 1 μ M isoproterenol ($C_m = 155$ pF). (B) Effect of 100 μ M ATP γ S and 1 μ M isoproterenol ($C_m = 110$ pF). In both experiments I_{Ca} was elicited by 200-ms depolarizations to 0 mV.

Effects of low doses of GTP γ S in the absence of GTP and of an ATP regenerating system. In the absence of phosphocreatine, ATP, and GTP (but in the presence of 1 mM MgCl₂ and 10 mM NaCl), intracellularly added GTP γ S was much more potent in increasing basal I_{Ca} . The addition of 100 or 25 μ M GTP γ S fully activated I_{Ca} within 5 min of perfusion (mean I_{Ca} densities were 12.3 ± 0.9 pA/pF, $n = 4$ and 12.1 ± 1.3 pA/pF, $n = 3$, respectively). The application of ATP γ S or isoproterenol was then unable to further increase I_{Ca} ; rather, ATP γ S had an inhibitory effect on I_{Ca} (not shown). At a concentration as low as 3 μ M, GTP γ S already markedly increased I_{Ca} ; the purinergetic stimulation was then limited so that I_{Ca} was 2.5 times larger than in control (Fig. 8 B). During the purinergetic stimulation I_{Ca} was not maintained and

further decreased during recovery. Another salient feature of these experiments was that the stimulatory effects of ATP γ S were not reproducible. A second application of ATP γ S was ineffective in increasing I_{Ca} but accelerated its rate of decrease. Isoproterenol application was without effect. Similar observations were obtained with two other cells. In two other cells, the addition of 1 μ M GTP γ S had no effect on basal I_{Ca} ; the external application of 100 μ M ATP γ S was still able to increase I_{Ca} by $77 \pm 2\%$ (not shown).

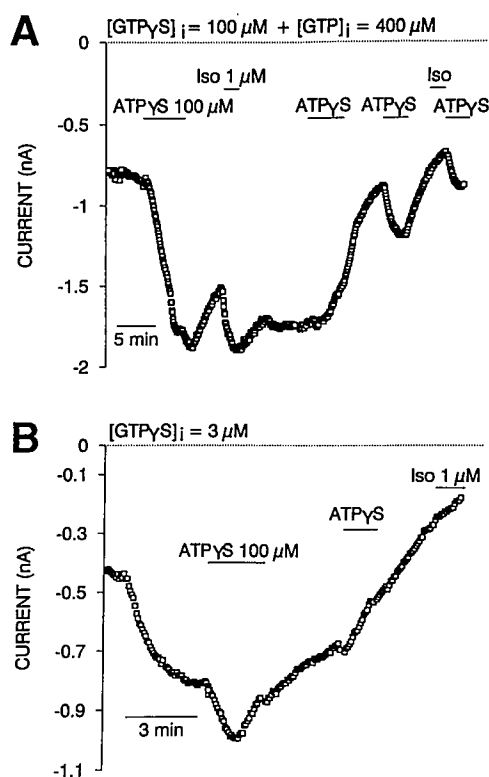


FIGURE 8. Effect of P₂-purinergic stimulation of I_{Ca} in the presence of low concentrations of GTP γ S. (A) The patch-pipette solution contained 100 μ M GTP γ S and 400 μ M GTP ($C_m = 130$ pF). (B) The patch-pipette solution contained 3 μ M GTP γ S but no GTP, ATP, or phosphocreatine. ($C_m = 85$ pF). In both cases I_{Ca} was elicited by 200-ms depolarizations to 0 mV.

Is a Phosphorylation Step Associated with the P₂-purinergic Stimulation?

To investigate whether a phosphorylation step was involved during the P₂-purinergic stimulation, the standard patch pipette solution contained 3 mM ATP γ S, which is a substrate for kinases but not for phosphatases. To favor the use of ATP γ S by kinases, ATP was omitted from the internal standard medium and total Mg²⁺ was decreased to keep a constant free Mg²⁺ concentration of 1 mM. Usually I_{Ca} did not significantly change during the internal perfusion of ATP γ S. Under this experimental condition, the external application of 100 μ M ATP γ S induced a pronounced increase in I_{Ca} amplitude which within 5–6 min reached a value similar to the one obtained in the presence of 100 μ M GTP γ S inside the cell ($134 \pm 11\%$ increase, $n = 5$) (Fig. 9A). The mean rate of increase was faster than under control conditions: 212 ± 27

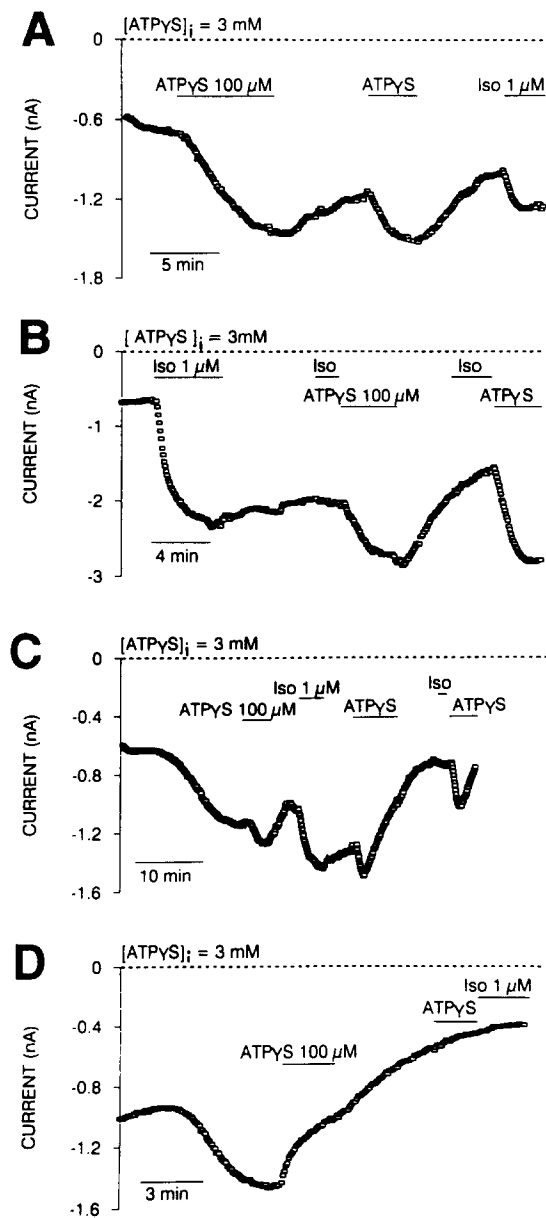


FIGURE 9. Effect of internal perfusion of ATP γ S on basal, P₂-purinergic, and β -adrenergic stimulated I_{Ca} . (A) Effects of successive applications of ATP γ S (100 μ M) and isoproterenol (*Iso*; 1 μ M). C_m = 115 pF. (B) Effects of successive applications of ATP γ S (100 μ M) after the current had been non-reversibly stimulated by isoproterenol (*Iso*; 1 μ M). Subsequent isoproterenol stimulations were ineffective. C_m = 105 pF. (C) Effects of successive applications of ATP γ S (100 μ M) and isoproterenol (*Iso*; 1 μ M) on a cell that exhibited an increase in basal I_{Ca} . C_m = 135 pF. (D) Lack of effects of ATP γ S (100 μ M) and isoproterenol (*Iso*; 1 μ M) on a cell that was perfused with a phosphocreatine-free solution. C_m = 130 pF. In each case, I_{Ca} was elicited by 200-ms depolarizations to 0 mV.

pA \cdot min⁻¹ (n = 5, P < 0.05). Washout of the agonist allowed I_{Ca} to recover. Isoproterenol had no effect when applied before I_{Ca} had begun to recover (not shown). However, when applied with a delay after ATP γ S removal, isoproterenol could stimulate I_{Ca} in a manner proportional to the decrease of I_{Ca} . As previously reported with GTP γ S, the β -adrenergic stimulatory effect was observed only once, whereas the P₂-purinergic stimulation was reproducible (Fig. 9 A).

In a second series of experiments performed under similar conditions (3 mM ATP γ S), application of 1 μ M isoproterenol induced an irreversible increase in I_{Ca} (Fig. 9 B). Note that this increase ($196 \pm 23\%$, $n = 3$) appears larger than under control conditions ($120 \pm 10\%$, $n = 12$; see Scamps et al., 1990b). P₂-purinergic stimulation of such a large, irreversibly isoproterenol-stimulated I_{Ca} induced a further increase in I_{Ca} ($39.1 \pm 4.5\%$; i.e., an increase that was 1.2-fold control I_{Ca} , $n = 3$). The purinergic-induced increase in I_{Ca} was reversible and reproducible, suggesting that phosphorylation was not involved in the purinergic effect. Note that on removal of ATP γ S, I_{Ca} decreased below the sustained isoproterenol-stimulated steady level.

In three cells the internal perfusion with 3 mM ATP γ S induced, after an 8–12-min delay, a slow increase in basal I_{Ca} ($69.4 \pm 3.9\%$, $n = 3$). When a steady state was reached after ~ 20 min, the external application of 100 μ M ATP γ S induced a further small increase in I_{Ca} amplitude which was followed by a pronounced decrease on washout (Fig. 9 C). The first application of isoproterenol enhanced I_{Ca} to an amplitude larger than the one reached after P₂-purinergic stimulation. This β -adrenergic sustained increase in I_{Ca} was reversed by ATP γ S application. Later, when I_{Ca} had reached its new steady-state amplitude, further applications of ATP γ S could transiently increase I_{Ca} ; isoproterenol could not.

A rather different situation was found when phosphocreatine and ATP were both omitted from the patch pipette solution. First, basal I_{Ca} increased more consistently under this experimental condition in the presence of internal ATP γ S; after a steady state was reached, the purinergic stimulation was without positive effect and induced only an inhibition of I_{Ca} . A second major observation was the absence of reproducibility of the purinergic stimulation (Fig. 9 D). Isoproterenol was without effect. Similar observations were found in two other cells.

Effect of Cholera Toxin

To further analyze the metabolic pathways involved during purinergic stimulation and particularly the role of G proteins, several toxins known to alter their properties were used. In a previous study (Scamps et al., 1990a), we have shown that P₂-purinergic stimulation was pertussis toxin insensitive.

When myocytes were incubated for at least 4 h with 10 μ g/ml cholera toxin, a toxin known to ADP-ribosylate the G_s proteins, basal I_{Ca} density was much larger than in control cells (Table I and Fig. 10). Application to these cells of 1 μ M isoproterenol or 10–100 μ M ATP γ S failed to further increase I_{Ca} , but ATP γ S still shifted the I - V curve and the availability curve (Figs. 10 and 11). The shift of the I - V curve was not statistically different, but the shift in the availability curve was significant (V_h at 50% inactivation was -27.2 ± 0.4 and -30.2 ± 0.4 mV, $n = 4$ in cholera toxin-treated cells and in the presence of 10 or 100 μ M ATP γ S, respectively, $P < 0.01$). ATP γ S at 10 or 100 μ M had no inhibitory effects on cholera toxin-treated cells.

To ensure that the P₂-purinergic stimulation was mediated through activation of the G_s protein, SDS-PAGE and autoradiograms of ³²P-labeled membrane proteins after ADP-ribosylation by cholera toxin were performed. At least two substrates for the ADP-ribosyltransferase of cholera toxin are reported in rat cardiomyocyte sarcolemmal membranes (Murakami and Yasuda, 1986; Foster et al., 1990). In the absence of cholera toxin, the autoradiograms reveal a weak radiolabeling of a 44-kD

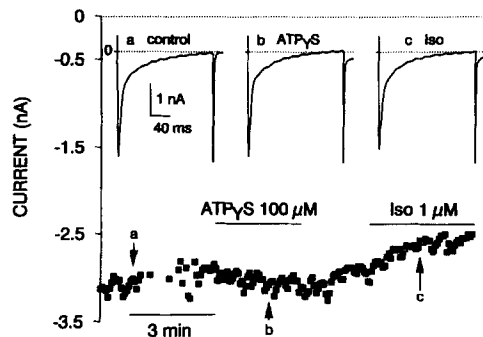


FIGURE 10. Effects of P_2 -purinergic and β -adrenergic stimulations of I_{Ca} on cholera toxin-treated cells. (Inset) Ca current traces under external ATP γ S and isoproterenol ($C_m = 170$ pF).

membrane protein, probably resulting from an endogenous ADP-ribosyltransferase (Fig. 12 *A, 1*). In the presence of cholera toxin, a marked increase in the 44-kD protein ADP ribosylation and some ADP ribosylation of a 47-kD protein were observed (Fig. 12 *A, 2*). Radiolabeling of the 44- and 47-kD proteins was GppNHP dependent (data not shown). Further identification of the G_s proteins was achieved by the use of anti- G_{sa} antiserum (Foster et al., 1990). The immunoblot shown in Fig.

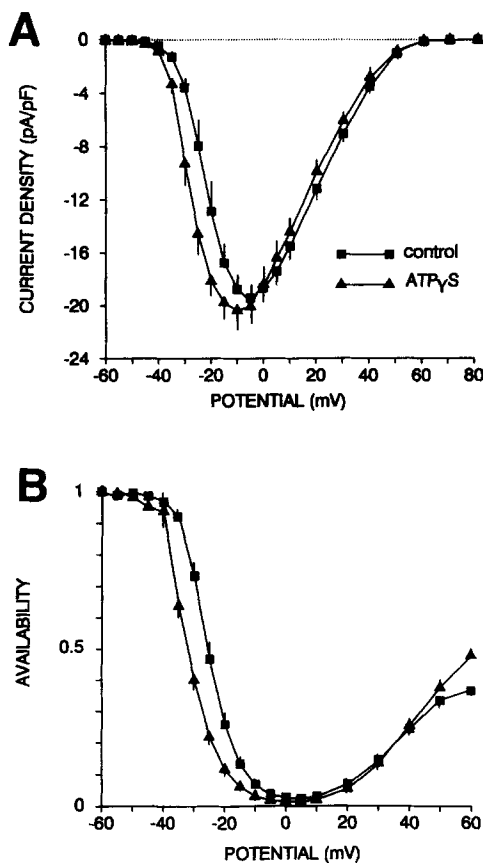


FIGURE 11. Effect of P_2 -purinergic agonist on I_{Ca} characteristics of cholera toxin-treated cells. (A) Peak Ca density-voltage relationships established on a cholera toxin-treated cell in the absence (\blacksquare) and in the presence (\blacktriangle) of ATP γ S; mean \pm SEM ($n = 4$). (B) Availability curves obtained on control cholera toxin-treated cells (\blacksquare) and in the presence of ATP γ S (\blacktriangle); mean \pm SEM ($n = 4$). Shift was significant (see text).

12 *B* reveals that the antiserum specifically recognized both the 44- and 47-kD proteins. Therefore, based on electrophoretic mobility, immunochemical properties, and susceptibility to cholera toxin, these two proteins appear to represent α subunits of G_s (G_{sa44} and G_{sa47} ; Northup, Sternweis, Smigel, Schleifer, Ross, and Gilman, 1980). Preincubation of cardiomyocytes for 3 min with 10 μ M isoproterenol led to a 40% reduction of the cholera toxin-dependent ADP ribosylation of G_{sa44} . Preincubation for 5 min with 100 μ M ATP γ S had a similar effect, with a 23% inhibition of G_{sa44} labeling. These results suggest that, like the β -adrenergic agonists, the P_2 -purinergic agonists act through activation of a G_{sa44} protein. Unfortunately, due to its low distribution it was not possible to evaluate to what extent G_{sa47} was affected by these agonists.

Cyclic AMP Measurements

In a previous report we suggested that the P_2 -purinergic increase in I_{Ca} was independent of Ca channel phosphorylation by cyclic AMP-dependent PKA since it was additive to a maximal stimulation by β -adrenergic agonists or internal cyclic AMP

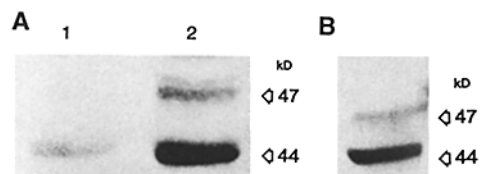


FIGURE 12. Characterization of the G_s proteins. (A) Cholera toxin-dependent ADP-ribosylation of the 44/47-kD proteins in rat crude membranes. ADP-ribosylation was performed in the absence (1) or in

the presence (2) of activated cholera toxin. The [32 P]ADP-ribosyl bound to 44-kD cholera toxin substrate in cardiomyocyte membranes was 704 ± 10 , 423 ± 33 , and 541 ± 29 fmol/mg protein in control, 10 μ M isoproterenol, and 100 μ M ATP γ S, respectively (mean \pm SEM, $n = 3$). (B) Immunoblot of rat cardiomyocyte membranes (100 μ g) with G_{as} peptide antiserum 584. Antiserum directed toward a specific region of G_{as} reacted with two bands (44 and 47 kD).

perfusion. Moreover, it was not prevented by a nonspecific inhibitor of phosphodiesterases, IBMX (Scamps et al., 1990a). However, the results presented above show that P_2 -purinergic as well as β -adrenergic stimulation is associated with an activation of the G_s proteins.

To confirm that the P_2 -purinergic stimulation did not induce an increase in cyclic AMP levels, measurements of cyclic AMP concentration were done in isolated rat ventricular cells. Aliquots of $\sim 100,000$ cells were first incubated with 100 μ M IBMX for 5 min to inhibit the phosphodiesterases; 10 μ M isoproterenol, 100 μ M ATP γ S, or 10 μ M isoproterenol plus 100 μ M ATP γ S was then added to cell batches for 5 min. The levels of cyclic AMP were 23.4 ± 0.8 , 138 ± 17.5 , 30.5 ± 1.8 , and 125 ± 8.6 pmol/mg protein (mean \pm SEM, $n = 9$ from three hearts), respectively, in control or in the presence of isoproterenol, ATP γ S, or isoproterenol plus ATP γ S. No significant increase in cyclic AMP level was observed with the P_2 -purinergic agonist, and P_2 purinoagonists have no antagonistic effect on cyclic AMP production by the supra-maximal activation of the β -adrenergic receptors with 10 μ M isoproterenol.

Cyclic GMP Measurements

Adenosine, a P₁-purinergic agonist, was previously reported to increase intracellular cyclic GMP concentration in isolated frog ventricle (Singh and Flitney, 1980) but not in guinea pig atria and ventricle (Brückner, Fenner, Meyer, Nobis, Schmitz, and Scholz, 1985). We thus investigated the effects of extracellular ATP γ S on the cyclic GMP level in isolated rat ventricular cells. In the presence of 100 μ M IBMX, basal cyclic GMP concentration was 272 ± 13 fmol/mg protein ($n = 5$ from two hearts). Incubation of the cells for 5 min with a solution containing 100 μ M ATP γ S increased the intracellular cyclic GMP content threefold (651 ± 65 fmol/mg protein, $n = 5$).

DISCUSSION

These results confirm that extracellular ATP in the micromolar concentration range increases the Ca current of cardiac muscle. They demonstrate that neither PKC activation nor the products of arachidonic acid metabolism were involved in the increase of I_{Ca} . Such an increase was also not consequent to cyclic AMP production, in agreement with recent studies performed in rat and mouse ventricular cells (Yamada, Hamamori, Akita, and Yokoyama, 1992; Zheng, Christie, De Young, Levy, and Scarpa, 1992). Interestingly, in the study of Yamada et al. (1992) measurements of cyclic AMP were done in the presence of a cyclic AMP phosphodiesterase inhibitor instead of the nonspecific phosphodiesterase inhibitor (IBMX). Under their experimental condition, ATP γ S induced a decrease in cyclic AMP levels which could be attributed to the increase in cyclic GMP levels that we report, assuming a cyclic GMP-dependent phosphodiesterase was activated. Despite its rather slow development, the ATP-induced increase in Ca current can be attributed to a direct activation of the Ca conductance by a G protein. P₁-purinergic stimulation of a cardiac K conductance is achieved similarly by a G protein (Kurachi et al., 1986). Moreover, based on electrophoretic mobility, immunochemical properties, and susceptibility to cholera toxin, it is proposed that the activating G protein is similar to the G_s protein involved during β -adrenergic stimulation which acts indirectly upon the Ca current by activation of adenylyl cyclase.

Besides increasing the Ca current, the P₂-purinergic stimulation induces a 5–10-mV shift in the hyperpolarizing direction of the activation curve and the current availability curve. Such shifts are observed on adding ATP γ S to the external solution under all of the experimental conditions we used (control, GTP γ S, and cholera toxin). Extracellularly applied ATP γ S as used in this study is roughly equipotent to ATP on the P₂-purinergic receptor (Scamps et al., 1990a) but much less on the P₃-purinergic receptor known to induce internal acidosis (Scamps and Vassort, 1990; Puc at et al., 1991b). This shift was also observed in Mg-free solution, a condition that definitively prevented activation of the P₃-purinergic receptors. Thus, the shift in I_{Ca} /voltage characteristics could not be related to a change in internal pH. This shift is consistent with an increase in negative charges on the extracellular face of the membrane after adsorption of ATP.

Involvement of a G Protein

The experiments performed with the poorly hydrolyzable analogues of GTP show that, in rat ventricular cells, a trinucleotide exchange of the G proteins may take place even in the absence of agonist, particularly when GTP γ S is used. The result of this exchange is an increase in basal I_{Ca} amplitude, which suggests either that the number of the stimulatory G proteins largely prevails over the inhibitory G proteins or that the stimulatory G proteins have a faster basal trinucleotide exchange rate than the inhibitory G proteins. Such an effect of poorly hydrolyzable analogues of GTP on basal I_{Ca} was also observed in guinea pig ventricular cardiomyocytes (Hescheler, Kameyama, and Trautwein, 1986; Shuba et al., 1990) but not in frog atrial cells (Breitwieser and Szabo, 1985; Nakajima, Wu, Irisawa, and Giles, 1990). In the presence of GppNHp or at a low GTP γ S/GTP ratio, I_{Ca} is hardly increased, a result that is quite consistent with competition between GTP and its hydrolysis-resistant analogues for the G proteins with an order of relative effectiveness GTP γ S > GTP > GppNHp (Yamanaka, Eckstein, and Stryer, 1986; Breitwieser and Szabo, 1988). This suggests that the cells can be depleted of GTP with great difficulty. In agreement with this proposal, we have shown that concentrations of GTP γ S as low as 3 μ M, in the absence of a GTP-regenerating system, could induce a twofold increase in I_{Ca} (see below, involvement of nucleoside diphosphate kinase). The experiments performed with GDP β S are also consistent with this proposal. Indeed, to block half of the P₂-purinergic and β -adrenergic stimulations, it was not only necessary to decrease the level of metabolites but also to decrease the level of free Mg²⁺ ions, a cofactor known to increase the affinity of G proteins for GTP but not for GDP (Gilman, 1987).

It is remarkable that the Ca current density could be increased to the same level (20 pA/pF, about threefold higher than the control value) by different means: cholera toxin pretreatment, internal perfusion with GTP γ S, or cumulative P₂-purinergic and β -adrenergic stimulation (Table I). This could mean that this value represents the maximal Ca current producible by these cells, or that GTP γ S and cholera toxin treatments allow maximal stimulation by both cascades involved in these stimulations. That the P₂-purinoceptor-induced increase in I_{Ca} is mediated through a G protein is supported particularly by its marked reduction in the presence of GDP β S (an approximately two times smaller increase than in control) and by the doubled and faster I_{Ca} increase induced by a maximal concentration of the agonist in the presence of weak concentrations of GTP γ S (together with GTP). The latter result suggests that the implied G protein possesses a rather strong GTPase activity and/or a rather slow turnover.

Identification of the Activatory G Protein

The high I_{Ca} density and the lack of effect of isoproterenol on cholera toxin-treated cells were expected since this toxin is known to activate the G_s proteins (Northup et al., 1980). Such an activation might lead to both a production of cyclic AMP and a direct stimulatory effect of Ca channels (Yatani et al., 1987, 1988; Brown and Birnbaumer, 1988; Yatani and Brown, 1989; Trautwein and Hescheler, 1990). The

involvement of a cholera toxin-sensitive G protein (namely, G_s protein) in the P₂-purinergic pathway was strengthened by demonstrating that cholera toxin-dependent ADP-ribosylation of the α_{s44} subunit of the G_s protein is reduced after β -adrenergic stimulation and also after P₂-purinergic stimulation, although to a slightly less extent. However, the different types of stimulation cannot involve the same pool of G_s proteins because their subsequent effects are specific and different; namely, an increase in cyclic AMP production and an irreversible effect of isoproterenol under GTP γ S or ATP γ S versus no measurable cyclic AMP production and a reversible and reproducible effect of purinergic stimulation under the same experimental conditions. This specificity of response implies either a spatial distribution and colocalization of the G_s protein with the two receptor types or that the two receptors are coupled to specific G_s proteins as shown for other G proteins (Kleuss, Hescheler, Ewel, Rosenthal, Schultz, and Witting, 1991). The latter explanation could result from splice variants of the α_s subunit (Mattera, Graziano, Yatani, Zhou, Graf, Codina, Birnbaumer, Gilman, and Brown, 1989) not resolved by electrophoretic mobility in our SDS-denaturing conditions or from specificity induced by at least one of the four isoforms of the $\beta\gamma$ dimer (Birnbaumer, Abramowitz, and Brown, 1990). It was also reported that cardiac G_s protein exists not only in particulate fraction (membrane) but also in soluble fraction (cytosol); this latter form is not efficient in activating adenylyl cyclase (Urasawa, Leiber, Roth, Hammond, and Insel, 1991).

Involvement of a Nucleoside Diphosphate Kinase

Surprisingly, in the presence of poorly hydrolyzable analogues of either GTP (GXP) or ATP, the purinergic-induced increase in I_{Ca} is reversible and can be reproduced. This is markedly different from the sustained β -adrenergic stimulation, which could be attributed to poorly reversible binding of α -GTP γ S leading to an elevated cyclic AMP production or to hydrolysis-resistant thiophosphorylation of the Ca channel (see below). This accounts for the fact that isoproterenol was active only on the first application. Assuming that GXP has activated all the G proteins during the ATP application, reproducibility of the purinergic effects implies that some G-GDP complexes are made available upon agonist removal, or that P₂-purinergic stimulation facilitates the GXP/GTP exchange (Otero, Li, and Szabo, 1991; Hilf, Kupprion, Wieland, and Jakobs, 1992), both conditions requiring endogenous GTP to be available. Since no GTP was added in the experiments performed with high concentrations of GXP, we have to assume that a GTP regenerative system should be present. A good candidate is the nucleoside diphosphate kinase (NDPK), for which the preferential phosphate donor is ATP (Otero, Breitwieser, and Szabo, 1988; Heidbüchel, Callewaert, Vereecke, and Carmeliet, 1990; Otero, 1990). Moreover, it has been shown that the membrane-associated NDPK is extractable together with a G_s protein as a complex form (Kimura and Shimada, 1988). Indeed, in the experiments designed to lower the internal ATP concentration (no added phosphocreatine or ATP) to tentatively reduce the NDPK activity, we failed to observe any reproducible stimulatory effects of the P₂-purinergic agonist (Figs. 8 B and 9 D). Moreover, experiments performed in the presence of internal ATP γ S (Fig. 9) allows us to postulate that the lack of reproducibility was not due to depletion of ATP per se.

Thus, it is proposed (see Fig. 13) that besides the GXP added to the pipette solution, and which is diffused to the membrane or translocated to the membrane through the NDPK, GTP is also made locally available by the NDPK from ATP whenever ATP is added or newly formed due to the creatine kinase activity when phosphocreatine is present. This implies a channeling of high energy phosphate from phosphocreatine to GDP by a cascade of two enzymes in which phosphocreatine and creatine kinase serve as an NTP-regenerative system while NDPK will use the newly formed NTP to restore GTP.

Phosphorylation

In the absence of agonists, intracellular perfusion of rat cardiomyocytes with ATP γ S can elicit a progressive increase in the L-type Ca current (Fig. 9 C) as already observed in guinea pig cells (Kameyama, Hescheler, Hofmann, and Trautwein, 1986). These effects were attributed to a basal turnover of phosphorylation/dephosphorylation of the Ca channel protein. Another interpretation of an intracellular

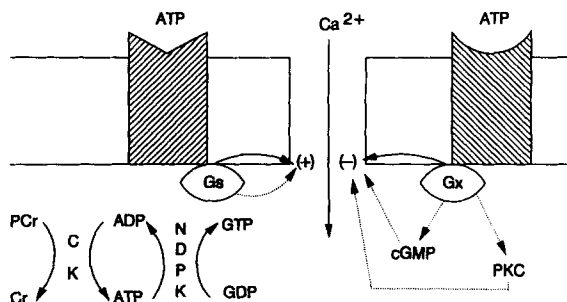


FIGURE 13. Schematic diagram illustrating the possible pathways leading to activation and inhibition of the Ca conductance. In both cases, a G protein seems to be required. Activation involves a G_s isoform most probably directly coupled to the Ca channel, although some indirect coupling not requiring phosphorylation cannot

be excluded. GTP is provided close to this G protein by an NDPK and a creatine kinase (CK). Inhibition also involves a G protein coupled directly to Ca channel or, more probably, a negative phosphorylation could be expected from activation of the PKC or of a cyclic GMP-dependent protein kinase (increase in cyclic GMP level).

ATP γ S-induced increase in current should be taken from the similar increase of the muscarinic K channel current in frog atrial cells (Otero et al., 1988), where the effects of ATP γ S were consequent to its conversion into GTP γ S by a nucleoside diphosphate kinase; the resulting increase in GTP γ S was assumed to be responsible for the sustained, direct activation of the K conductance. Moreover, a longer delay in the presence of ATP γ S compared with GTP γ S was necessary to see an effect, which was consistent with an intermediary metabolic step. That such an ATP γ S to GTP γ S conversion has occurred during our experiments could account for the slowly developing increase in I_{Ca} seen in few cells before agonist application (Fig. 9, C and D). On internal perfusion of ATP γ S, the increase in basal I_{Ca} required at least 8–10 min, a value similar to the one reported by Otero et al. (1988). Besides, when no apparent effect on basal I_{Ca} was observed, application of the P₂-purinergic agonist induced effects quite similar to those obtained with the low GTP γ S concentration (i.e., potentiation, reversibility, and reproducibility). As with the slowly hydrolyzable analogues of GTP, the repeatability of P₂-purinergic-induced increase in I_{Ca} was

suppressed when phosphocreatine was omitted, i.e., when no ATP was provided to the NDPK reaction. Under this condition the NDPK utilizes only the internal ATP γ S, leading to formation of GTP γ S, potentiation of the P₂-purinergic stimulation, and development of the G protein-dependent inhibition. Incidentally, the sustained β -adrenergic increase in I_{Ca} in the presence of internal ATP γ S might result from both thiophosphorylation of the Ca channel and sustained activation of the adenylyl cyclase by α_s -GXP.

Inhibition of I_{Ca}

In the presence of hydrolysis-resistant GTP analogues, the increase in I_{Ca} is not maintained during prolonged or repetitive external applications of ATP γ S (Figs. 7 and 8). A slow decrease occurs which could be attributed to the activation of an inhibitory pathway in addition to the GXP/GTP exchange described above. Such an inhibitory effect was also observed under control conditions in mammal cells (Qu, Campbell, Whorton, and Strauss, 1991) or with high ATP concentrations in frog cells (Alvarez et al., 1990). The reduction of I_{Ca} cannot be attributed to activation of P₁ purinoceptors since it is neither mimicked by adenosine nor prevented by IBMX (P₁ purinoceptor antagonist). The latter experiment also suggests that the inhibitory effect does not result from phosphodiesterase activation leading to cyclic AMP degradation. Moreover, the lack of effects of forskolin allows us to postulate that the inhibitory effect is not due to a direct inhibition of adenylyl cyclase through a G_i protein. In addition, the reported increase in cyclic GMP cannot be attributed to activation of P₁-purinergic receptors since it was observed in the presence of IBMX. Thus, we can propose that the negative effect of ATP γ S is consequent to P₂-purinergic stimulation, although we have not yet established which subtype is involved (P_{2X} or P_{2Y}), and that this negative effect does not appear to be linked to cyclic AMP production or degradation. Interestingly, in ferret heart cells ATP and its derivatives had only a negative effect on I_{Ca} amplitude, which was attributed to P_{2Y} purinoceptor stimulation (Qu et al., 1991).

Several mechanisms can account for this decrease in I_{Ca} and may be related to a phosphorylation of an inhibitory site of the Ca channel protein (Fig. 13). Purinergic stimulation increases the cyclic GMP level. It might thus activate the cyclic GMP-dependent protein kinase (PKG) that has been reported to inhibit I_{Ca} in several tissues, including cardiac cells (Méry, Lohman, Walter, and Fischmeister, 1991). Purinergic stimulation also increases the phosphoinositide turnover and thus might activate the Ca- and phospholipid-dependent PKC. Activation of PKC by phorbol esters reduces I_{Ca} (Tseng and Boyden, 1991) and reduces the open probability of the Ca channel after a transient increase (Lacerda, Rampe, and Brown, 1988), although the same authors reported that there was no effect on Ca current under whole-cell patch-clamp conditions. One cannot exclude another possibility that sustained activation of a G protein would have a direct inhibitory effect on the cardiac I_{Ca} as reported for the N-type Ca channel in neuronal cells (Dolphin, 1990). Whatever the exact mechanism of inhibition, these effects were clearly observed only in the presence of hydrolysis-resistant GTP analogues; this suggests that this inhibitory effect also occurs through activation of a G protein and might involve an hydrolysis-resistant phosphorylation.

Kinetic Aspects

From studies relating $I_{K(ACh)}$ (Breitwieser and Szabo, 1985) and isoproterenol-stimulated I_{Ca} (Yatani and Brown, 1989), the idea has emerged that ion channel regulation through the membrane-delimited G protein pathway is a rather fast process. However, a slow rate of increase as seen during P_2 -purinergic stimulation might not necessarily involve a multistep pathway. As reviewed by Gilman (1987) and Levitzki (1988), the kinetic features of the hormone-G protein-effector complex depend on the agonist-receptor interactions, on the intrinsic nature of the G protein, and on the G protein-effector interactions. Thus, a low density of P_2 purinoceptors as yet unknown or a weak interaction of this G_s isoform with the Ca channel could be rate limiting.

That the increase in I_{Ca} continued to develop after brief isoproterenol application (see Fig. 3) can be accounted for by the slow GTPase step as compared with the turnover number of activated cyclase; ~ 100 cyclic AMP molecules can be produced before the GTPase-off step (Levitzki, 1988). Such is not the case with P_2 -purinergic stimulation, which is consistent with the concept of a direct G protein-Ca channel coupling. Further support for this interpretation is given by the fact that in the presence of a low GTP γ S concentration the rate and amplitude of P_2 -purinergic increase in I_{Ca} were enhanced (see Fig. 8), in agreement with the fact that the poorly hydrolyzable analogue forms a more stable G_s protein-Ca channel complex.

Conclusions

The present results suggest that P_2 -purinergic stimulation would both activate and more slowly inhibit the Ca current. Activation is suggested to involve a direct coupling of the Ca channel with a G_s protein which is in some ways different from the G_s protein activating the adenylyl cyclase. P_2 -purinergic activation of I_{Ca} would thus have similarities with the direct activation by a G protein of the K conductance under adenosine-mediated P_1 -purinergic stimulation. Besides, several inhibitory pathways are tentatively proposed. Moreover, our results suggest that a nucleoside diphosphate kinase can be involved to channel high energy phosphate to the GTP used by the G_s protein associated with the Ca channel.

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