Intracellular ATP slows time-dependent decline of muscarinic cation current in guinea pig ileal smooth muscle

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Inoue, Ryuji, and Yushi Ito. Intracellular ATP slows time-dependent decline of muscarinic cation current in guinea pig ileal smooth muscle. Am J Physiol Cell Physiol 279: C1307–C1318, 2000.—The effects of intracellular nucleotide triphosphates on time-dependent changes in muscarinic receptor cation currents (Icat) were investigated using the whole cell patch-clamp technique in guinea pig ileal muscle. In the absence of nucleotide phosphates in the patch pipette, Icat evoked every 10 min decayed progressively. This decay was slowed dose dependently by inclusion of millimolar concentrations of ATP in the patch pipette. This required a comparable concentration of Mg2+, was mimicked by UTP and CTP, and was attenuated by simultaneous application of alkaline phosphatase or inhibitors of tyrosine kinase. In contrast, a sudden photolytic release of millimolar ATP (probably in the free form) caused a marked suppression of Icat. Submillimolar concentrations of GTP dose dependently increased the amplitude of Icat as long as ATP and Mg2+ were in the pipette, but, in their absence, GTP was ineffective at preventing Icat decay. The decay of Icat was paralleled by altered voltage-dependent gating, i.e., a positive shift in the activation curve and reduction in the maximal conductance. It is thus likely that ATP exerts two reciprocal actions on Icat, through Mg2+-dependent and -independent mechanisms, and that the enhancing effect of GTP on Icat is essentially different from that of ATP.

ATP IS INVOLVED IN A VARIETY of fundamental cellular reactions associated with, e.g., synthesis of many bioactive molecules, performance of mechanical work, and active and passive transport of molecules and ions. Modification of ionic channel activity by ATP is a widespread mode of regulating cellular functions. For instance, phosphorylation of channel protein and associated regulatory subunits by protein kinases leads to altered kinetics of activation, inactivation, or desensitization in many different types of channel (24). In some, ATP is required through its hydrolysis, whereas in others it inhibits the channel activity through direct interaction with intracellular sites [ATP-sensitive K+ channels (31, 34); voltage-dependent Ca2+ channels, (17, 28, 41); Ca2+-activated nonselective cation channels (33, 35); ion transporters and exchangers (10)].

The functional significance of Icat regulation by intracellular ATP and other high-energy phosphates has recently been investigated in some detail. In the guinea pig gastric muscle, phosphorylation of the muscarinic receptor by protein kinase C, Ca2+/calmodulin, and myosin light chain kinase has been implicated in desensitization and maintenance of Icat activity (18, 19, 21). In guinea pig ileal muscle, a strong enhancing effect of phosphocreatine and an inhibitory effect of ATP were observed on Icat, and the sites of these actions appeared to be downstream of the receptor (1). However, whether protein phosphorylation is involved in these effects remains equivocal, and, more importantly, the results of this study seem inconsistent in several critical points with those of previous studies including our own, particularly as to the actions of ATP, GTP, and Mg2+ (15, 42; for details see DISCUSSION).

The goal of the present study was therefore twofold. First, we sought appropriate conditions for assessing the efficacy of intracellular perfusion on Icat. This was...
essential, since no single channel recording of $I_{\text{cat}}$ channels in the inside-out patch configuration has yet been published, due to their rapid rundown on patch membrane excision. Second, we reevaluated the roles of nucleotide phosphates such as ATP and GTP under these conditions. The results of our work indicate 1) that intracellular ATP has dual actions on $I_{\text{cat}}$ in a Mg$^{2+}$-dependent and independent fashion and 2) that the intracellular GTP concentration is a critical determinant of $I_{\text{cat}}$ activity in a manner independent of ATP, although both nucleotide phosphates can modify the gating properties of $I_{\text{cat}}$ channels in a similar way.

**METHODS**

**Cell Isolation**

The procedures used for cell isolation are essentially the same as described previously (38). Guinea pigs of either sex (500–1,000 g) were killed quickly by exsanguination. After opening the abdominal cavity, an ~5-cm-long segment of intestine (5–10 cm proximal from the ileocecal valve) was rapidly excised. A glass pole (10 cm in length and ~2 mm in diameter) was inserted into the segment, which was tied onto it at both ends with a silk thread, and incubated successively in nominally Ca$^{2+}$-free Krebs solution and a solution containing 1.5 mg/ml collagenase (Sigma type I) at 35°C for 5 and 20 min. Thereafter, the digested segment was cut open and stored in a refrigerator until use. Just before each experiment, the longitudinal muscle layer was peeled off from the remainder of ileum using two fine forceps and minced into small pieces. Single cells were mechanically dispersed by agitating these pieces using a blunt tipped Pasteur pipette.

The temperature of the superfusing solution was strictly controlled using a commercial warmer unit (TC-344B; Warner Instruments; accuracy ±0.5°C). This was necessary because the activity of $I_{\text{cat}}$ is profoundly affected by the ambient temperature, as shown in Fig. 1C. All experiments were performed at 25°C, unless otherwise stated.

**Electrophysiology**

The system used for patch-clamp experiments was essentially the same as described previously (38). Cell capacitance (56.7 ± 0.7 pF, $n = 270$) and 50–80% of series resistance or access resistance $R_a$ (10.7 ± 2 MΩ, $n = 145$) were electronically compensated. The liquid junction potential arising at the interface between a patch pipette and the bath (~6 mV; Ref. 38) was corrected when current-voltage relationships were constructed. In some experiments where rapid switching of external solution was needed (see Fig. 6A), a solenoid-controlled fast solution exchange device was employed (DAD-12C; Scientific Instruments).

$[\text{Ca}^{2+}]_i$ was clamped close to a resting value typical for gut smooth muscle cells (~100 nM; e.g., Ref. 29) to minimize the influence of changes in $[\text{Ca}^{2+}]_i$ on $I_{\text{cat}}$ (13, 29). A mixture of 10 mM BAPTA and 5 mM CaCl$_2$ was used for this purpose, since BAPTA is superior to EGTA in buffering $[\text{Ca}^{2+}]_i$ to an almost constant level with varying concentrations of nucleotide phosphates and Mg$^{2+}$ (see Table 1).

To minimize the desensitization of $I_{\text{cat}}$, the time of carbachol (CCh) application was limited to 20 s (which was, however, long enough to evoke the maximum response) and a recovery interval of 10 min was used, which were empirically determined according to the results of nystatin-perforated recordings. The amplitude of $I_{\text{cat}}$ was normalized by cell capacitance to eliminate variations arising from different cell sizes. Care was also taken to minimize the tissue-to-tissue variation in $I_{\text{cat}}$ activity, by comparing data between cells from the same batch for the same series of experiments.

**Flash Photolysis**

Caged ATP (8 mM) was introduced into the cell via a patch pipette, and brief control applications of CCh (100 μM) were made twice in the dark. A high-pressure mercury lamp (intensity 320 mW/10 mm$^2$ at 360 nm) was used to generate

![Fig. 1. Influence of access resistance ($R_a$) and temperature on cation current ($I_{\text{cat}}$) activity. Bath contained physiological salt solution (PSS). Holding potential ($V_h$), −60 mV. A: time course of $I_{\text{cat}}$ decay with no ATP in the patch pipette from 19 different cells with different $R_a$ values. For the sake of convenience, data are grouped depending on $R_a$, i.e., $R_a < 15$ MΩ (○) and $R_a > 20$ MΩ (●). Averaged current densities from each group are plotted against the time elapsed after establishing the whole cell configuration. Carbachol (CCh; 100 μM) was applied for 20 s every 10 min. *Statistically significant differences with unpaired t-test. B: relationship between $I_{\text{cat}}$ densities at 10 min (○) and 30 min (●) after establishing whole cell configuration. Data are from A. Lines are drawn according to the best linear fit of data points; $r$, correlation coefficients. C: relationship between $I_{\text{cat}}$ density and temperature. The pipette solution: 2ATP; $n = 8–18$. Inset: a typical example at 25 and 15°C recorded from the same cell.](http://ajpcell.physiology.org/)
ultraviolet (UV) light, which was applied through an electronic shutter (HB-10103AF; Nikon, Tokyo, Japan) controlled by a voltage pulse generator (SEN-7103; Nihon Kohden). Because the flash intensity of 20 mJ/10 mm² at 347 nm causes ~40% photolysis of 5 mM caged ATP in vitro, we assumed that 200 ms flash duration would be sufficiently long to degrade 8 mM caged ATP almost completely.

### Solution

Table 1 gives the detailed composition of internal solutions used in the present study, where free and bound concentrations of nucleotides, Mg²⁺, and Ca²⁺, except for test substances, are kept as constant as possible (3, 9). Bath solution (physiological salt solution (PSS)) contained (in mM) 140 Na⁺, 6 K⁺, 1.2 Mg²⁺, 2 Ca²⁺, 151.4 Cl⁻, 10 glucose, and 10 HEPES (adjusted at pH 7.4 with Tris base). To obtain Cs⁺ external solution, Na⁺ was isosmotically replaced by Cs⁺. Pipette solution contained (in mM) 140 Cs⁺, 20 Cl⁻, 120 aspartate, 10 HEPES, 10 BAPTA, and 5 Ca²⁺ (adjusted at pH 7.2 with Tris base). For other compositions see Table 1 or figure legends.

### Chemicals

ATP, GTP, UTP, CTP, ADP, AMP, and caged ATP [P²⁺-[1-(2-nitrophenyl)ethyl]adenosine-5'-triphosphate, trisodium salt] were purchased from Dojin, 5'-adenyllylimidophosphate (AMP-PNP), alkaline phosphatase, calf thymus, C, H⁻, KN-62, and okadaic acid were from Calbiochem, and FK506 was a kind gift of Dr. H. Onoue (Department of Pharmacology, Kyushu University, Fukuoka, Japan).

### Statistics

All results are expressed as means ± SE. Statistical significance of differences between given sets of data were evaluated by Student's t-test for single comparison and one-way ANOVA or Dunnett's tests for multiple comparison.

### RESULTS

#### Factors Affecting I_{cat} Activity

**Efficacy of internal perfusion.** When single guinea pig ileal smooth muscle cells were intracellularly perfused with Cs⁺ via a patch pipette, full development of voltage-dependent Ca²⁺ current was observed within a few minutes after establishment of the whole cell configuration (data not shown; cf. Ref. 22; ~2 min in urinary bladder). Similarly, Ca²⁺ chelators such as EGTA and BAPTA included in the patch pipette rapidly abolished a Ca²⁺-dependent component of outward rectifying K⁺ current (data not shown). These results strongly suggest that, despite the spindle-shaped geometry of ileal smooth muscle cells, equilibration of these substances between the pipette and cytosol was fast and comparable to simple diffusion (30). In contrast, when nucleotide phosphates were omitted from the pipette solution, the amplitude of I_{cat} changed with a much slower time course. For example, without ATP in the pipette, >20 min were needed for I_{cat} to decay almost completely after the start of whole cell configuration (Fig. 1A; see also Fig. 2Ab). The rate of I_{cat} decay was significantly slower in cells having large Rₓ values (>20 MΩ), compared with those with smaller Rₓ values (<15 MΩ; Fig. 1A). Accordingly, the I_{cat} density 30 min after internal perfusion was larger as the value of Rₓ increased (closed circles and dotted line in Fig. 1B). In contrast, no clear correlation was found between Rₓ and I_{cat} density for the first application of CCh, when the cell had already been dialyzed for 10 min (open circles and solid line in Fig. 1B). Essentially, the same observations were obtained when Mg²⁺ instead of ATP was eliminated from the pipette solution (data not shown).

**Influence of ambient temperature.** The temperature of the bathing solution also critically affected I_{cat} activity. As shown in Fig. 1C, a fall in temperature from 25 to 15°C caused an about threefold reduction (i.e., Q_{10} ≈ 3.0) in I_{cat} amplitude. This means that I_{cat} activity is highly susceptible to changes in the ambient temperature, which could fluctuate by >10°C within 1 day or between different seasons.

These observations strongly suggest that at least three factors, Rₓ, temperature, and time of agonist application, are crucial for determining I_{cat} activity. Thus, for the rest of this study, to minimize the influence of these factors, we adopted the following empip-
the amplitude of $I_{\text{cat}}$ remained almost constant over a period of 30 min. The rapid decay of $I_{\text{cat}}$ in the absence of ATP was usually accompanied by progressive cell contracture, even though the cell was initially relaxed and dialyzed with a high concentration of Ca$^{2+}$ buffer (10 mM BAPTA).

Figure 2B summarizes data pooled from 8–10 similar experiments, with respect to 10, 1, and 0 mM ATP included in the pipette (Mg$^{2+}$ present). Single exponential fitting of data revealed that the time course of $I_{\text{cat}}$ decay was significantly retarded by increasing the concentration of ATP in the pipette ([ATP]). The apparent time constant estimated from the averaged $I_{\text{cat}}$ decay was 7.6 min for 0 mM ATP, and this value was increased to 24.5, 32.8, and >50 min for 1, 2, and 10 mM ATP, respectively. Correspondingly, $I_{\text{cat}}$ density 30 min after the start of internal perfusion became significantly larger with higher [ATP], (Fig. 3A; $P < 0.05$ with one-way ANOVA).

The retarding effect of intracellular ATP on $I_{\text{cat}}$ decay is likely to be associated with the concentration of the MgATP complex rather than that of free ATP. First, under conditions in which the concentrations of Mg$^{2+}$, Ca$^{2+}$, and the free and bound forms of GTP were kept almost constant, the calculated [MgATP] is well correlated with $I_{\text{cat}}$ density 30 min after internal perfusion (Fig. 3A and Table 1). Second, reducing the Mg/ATP ratio while keeping the concentration of the MgGTP complex at a similar level (10ATP/2Mg) or rigorous chelation of Mg$^{2+}$ by 2 mM EDTA (10ATP/2EDTA) resulted in a marked reduction in $I_{\text{cat}}$ density (Fig. 3B; $P < 0.05$ with Dunn’s test).

We also tested the ability of other nucleotide phosphates such as CTP, UTP, GTP, GDP, ADP, and AMP to maintain $I_{\text{cat}}$. Among them, CTP and UTP exhibited a comparable efficacy to ATP (Mg$^{2+}$ required) in prolonging the time course of $I_{\text{cat}}$ decay (time constants >50 and 49.0 min, respectively; Fig. 3C). In contrast, GTP, GDP (not shown), ADP, and AMP were all virtually ineffective at preventing $I_{\text{cat}}$ decay when added alone in the pipette (Fig. 3C; $P < 0.01$ with Dunn’s test). These results indicate that the effect of ATP is not mediated by its metabolites such as ADP and AMP or through its conversion to GTP by adenylyl transferase.

ATP Hydrolysis Is a Prerequisite

The requirement of the MgATP complex for maintaining $I_{\text{cat}}$ activity suggests that some cellular process(es) that utilizes the hydrolytic energy of ATP may be involved. To test this, we performed the next series of experiments and obtained the following results (Fig. 3D).

First, the poorly hydrolyzable analog of ATP, AMP-PNP (2 mM), was totally ineffective at preventing $I_{\text{cat}}$ decay. Second, internal perfusion of the thiophosphate analog of ATP, adenosine 5'-O-(3-thiotriphosphate) (ATP[S]; 2 mM), resulted in induction of a small inward current by itself and sustained activation of $I_{\text{cat}}$ in response to subsequently applied CCh (Fig. 3D; see
also the inset). Third, simultaneous inclusion of alkaline phosphatase (100 μg/ml) with ATP in the pipette significantly reduced \( I_{\text{cat}} \) density 30 min after the start of internal perfusion. Fourth, no significant difference in \( I_{\text{cat}} \) density was observed between control cells and those perfused with a mixture of inhibitors for A, C, and G kinases and calmodulin-dependent kinase II (30 μM H-7, 5 μM KN-62, and 1 μM calphostin C). Fifth, two mechanistically distinct tyrosine kinase inhibitors, genistein (50 μM; Fig. 3D) and tyrphostin A-25 (not shown), greatly reduced \( I_{\text{cat}} \) density (16). Sixth, okadaic acid and FK506, at concentrations known to block types 1, 2A, and 2B phosphatases (2.5 μM; FK, FK506 (3 μM); CM, calmodulin (30 μM); CalC, calphostin C (1 μM); H7, H-7 (30 μM); KN62, KN-62 (5 μM); genistein (50 μM). Inset: an example of \( I_{\text{cat}} \) with 2 mM ATP/S in the pipette. Asterisks indicate statistically significant differences for 10ATP (B and C) and 2ATP alone (D) with Dunnett’s test (\( P < 0.05 \)), respectively.

These results collectively suggest that ATP hydrolysis is essential for maintaining \( I_{\text{cat}} \) activity and that tyrosine phosphorylation may play an integral role.

**Inhibitory Effect of ATP on \( I_{\text{cat}} \)**

It has previously been reported that the \( I_{\text{cat}} \) channel is subject to ATP-mediated inhibition (1). This is consistent with our present observation that \( I_{\text{cat}} \) density, evaluated on a statistical basis, was significantly smaller with 10 mM ATP and 2 mM Mg\(^{2+}\) (10ATP/2Mg) than with 2 mM ATP and 2.5 mM Mg\(^{2+}\) (2ATP; Fig. 3, A and B; \( P < 0.05 \) with unpaired \( t \)-test), where the concentration of free ATP is \( \sim 13 \) times higher in the former than the latter, while [MgATP] and [MgGTP] are almost the same (Table 1). To confirm
this possible inhibitory effect of free ATP on $I_{\text{cat}}$ more directly, we examined the effects of photolytically released ATP on the time course of $I_{\text{cat}}$ in the same cell.

As demonstrated in Fig. 4, UV flashes of a maximally effective duration (200 ms; see METHODS) produced little effect on $I_{\text{cat}}$ evoked by the continued application of CCh (100 \( \mu \text{M} \)), when caged ATP was not included in the pipette (2ATP\(^*\), Fig. 4A). In contrast, when 8 mM caged ATP was present, a single 200-ms flash, which is expected to increase free ATP concentration from 0.68 to \(~4\text{ mM}\) (compare 2ATP\(^*\) and 10ATP/2Mg in Table 1) for an estimated change in [ATP] before and after photolysis), caused a marked reduction in $I_{\text{cat}}$ amplitude (Fig. 4B; time constant \( \tau = 10.0 \pm 2.0 \text{ s} \), \( n = 15 \)). A subsequently applied flash was no longer effective. The effect of photolysis is unlikely to be due to the release of photoproducts other than ATP (i.e., iminodiacetic acid), since uncaging of caged inositol trisphosphate, which had the same caging moiety (nitrophenylacetic acid), did not affect $I_{\text{cat}}$ (data not shown). As summarized in Fig. 4D, an \(~50\%\) inhibition of $I_{\text{cat}}$ occurred on photolysis of 8 mM caged ATP (2ATP\(^*\) + 8 caged ATP). Remarkably, the inhibitory effect of photolysis was almost abolished, when free ATP concentration had already been raised to \(~4\text{ mM}\) (10ATP/2Mg + 8 caged ATP; Fig. 4D; see also Table 1). These results strongly suggest that free ATP rather than MgATP is responsible for this inhibition, which would saturate at free ATP concentrations of several millimolar. The degree of inhibitory effect of photolytically released ATP was not significantly changed when $I_{\text{cat}}$ was activated by internally applied guanosine 5’-O-(3-thiotriphosphate) (GTP\(\gamma\)S; 50 \( \mu \text{M} \)) with inhibitors of protein kinases A, C, and G and calmodulin kinase II, H-7 (30 \( \mu \text{M} \)), calphostin C (1 \( \mu \text{M} \)), and KN-62 (5 \( \mu \text{M} \)) (Fig. 4, C and D). These results suggest that the inhibitory effect of ATP may be exerted in a nonenzymatic fashion, presumably by acting directly on the $I_{\text{cat}}$ channel protein.

Intracellular GTP Potentiates $I_{\text{cat}}$ But Does Not Affect Its Slow Time-Dependent Decay

It has been suggested that persistent stimulation of muscarinic receptor in the presence of CCh causes progressive desensitization of $I_{\text{cat}}$ due mainly to depletion of intracellular GTP (43). We therefore investigated how intracellular GTP concentration ([GTP]\(_i\)) affects the time-dependent decay of $I_{\text{cat}}$ evoked intermittently by brief application of CCh, while keeping [MgATP] high enough to maintain $I_{\text{cat}}$ (note that Mg-GTP itself was ineffective; see Fig. 3C) and free ATP concentration, [Mg\(^2+\)], and [Ca\(^2+\)] as constant as possible (see Table 1) to exclude their indirect influence on $I_{\text{cat}}$ activity. When [GTP]\(_i\) was lowered to <1 mM (open circles and squares), the amplitude (or density) of $I_{\text{cat}}$ decreased rapidly toward a steady level, although the initial $I_{\text{cat}}$ density was similar regardless of [GTP]\(_i\) (Fig. 5A; \( P > 0.05 \) with one-way ANOVA). The time course of this decrease was not slowed by increasing [GTP]\(_i\) (time constant of decay: 4.3 and 5.8 min for 0.01

Fig. 4. Flash photolysis of caged ATP inhibits $I_{\text{cat}}$. A and B: CCh (100 \( \mu \text{M} \))-induced $I_{\text{cat}}$ in the absence (A) and presence (B) of caged ATP. Cs\(^+\) was used as the charge carrier in the external solution to augment the magnitude of $I_{\text{cat}}$ (42, 43). To allow the effects of internal perfusion to equilibrate, control applications of CCh were made twice in the dark before UV flashes were applied (i.e., 30 min after the start of internal perfusion). Caged ATP (8 mM) was added in the pipette solution, 2ATP\(^*\). C: guanosine 5’-O-(3-thiotriphosphate) (GTP\(\gamma\)S) induced $I_{\text{cat}}$ with 8 mM caged ATP and protein kinase inhibitors in the pipette (50 \( \mu \text{M} \) GTP\(\gamma\)S instead of GTP, 5 \( \mu \text{M} \) KN-62, 30 \( \mu \text{M} \) H-7, and 1 \( \mu \text{M} \) calphostin C were added in 2ATP\(^*\)). $V_H = -60$ mV. Flash stimuli (200 ms) were applied at arrows. D: summary of the effects of photolytically released ATP (\( n = 3–15 \)). From left to right: 2ATP\(^*\) alone, 2ATP\(^*\) plus 8 mM caged ATP, 10ATP/2Mg plus 8 mM caged ATP for CCh-evoked $I_{\text{cat}}$, 2ATP\(^*\) plus 8 mM caged ATP and protein kinase inhibitors (see above) for GTP\(\gamma\)S-induced $I_{\text{cat}}$. The amplitude of $I_{\text{cat}}$ 10 s after flashes was normalized to that immediately before them. The rate of $I_{\text{cat}}$ amplitude decline due to desensitization was as small as 5 ± 2% per 10 s (\( n = 6 \); measured from the peak) and was thus not corrected. Asterisks indicate statistically significant differences with paired \( t \)-test.
and 0.1 mM GTP, respectively) and appeared to be faster than that of $I_{\text{cat}}$ decay observed with low [ATP]; 7.6 and 24.5 min for 0 and 1 mM ATP, respectively; Fig. 2B), suggesting that different mechanisms may be involved. At the steady phase (30 min after internal perfusion), $I_{\text{cat}}$ density was an incremental function of [GTP], with an apparent $EC_{50}$ value of ~100 μM (Fig. 5B; $P < 0.05$ with one-way ANOVA). No further significant increase or decrease was observed at GTP concentrations >1.2 mM and <0.01 mM, respectively ($P > 0.05$ with pooled variance $t$-test).

These results clearly indicate essential differences in the actions of ATP and GTP on $I_{\text{cat}}$; intracellular GTP critically controls the steady-state activity of $I_{\text{cat}}$ but cannot prevent the time-dependent decay of $I_{\text{cat}}$ (see Fig. 2B), and vice versa for ATP.

Altered Receptor Sensitivity May Only in a Minor Way Contribute to $I_{\text{cat}}$ Decay

Time-dependent decay of agonist-induced responses has often been ascribed to reduced receptor sensitivity (25). However, as illustrated and summarized in Fig. 6, A and B, respectively, the relationship between CCh concentration and $I_{\text{cat}}$ amplitude was only modestly changed during the course of $I_{\text{cat}}$ decay. The apparent dissociation constant for CCh evaluated empirically by Hill analysis increased only slightly during the progression of internal perfusion (3 and 15 μM at 10 and 50 min, respectively). These results suggest that reduced receptor sensitivity, albeit present, would contribute only in a minor way to the time-dependent decay of $I_{\text{cat}}$.

$I_{\text{cat}}$ Decay Parallels the Negative Shift in Half-Activation Voltage and Reduction in Maximal Conductance

In the next series of experiments, we examined what changes occur in the properties of voltage-dependent gating of $I_{\text{cat}}$ during the course of $I_{\text{cat}}$ decay. Typical examples of current-voltage ($I-V$) relationships and steady-state activation curves at 10, 20, 40, and 60 min are displayed for a case in which 2 mM ATP was present in the pipette (2ATP; Fig. 7, A and B). As the decay proceeded, the peak of the U-shaped $I-V$ curve was shifted positively, and correspondingly the half-activation voltage ($V_{1/2}$) and maximal conductance ($G_{\text{max}}$) became more positive and smaller, respectively. In contrast, the reversal potential and the slope factor of the Boltzmann curve remained almost unchanged (Fig. 7, A and D). Figure 7, C and D, summarizes this type of experiment from eight different cells. Forty minutes after the onset of internal perfusion, $V_{1/2}$ increased by ~25 mV and $G_{\text{max}}$ decreased to ~0.4 of the initial value (open symbols in Fig. 7C). The extent of reduction in $I_{\text{cat}}$ density expected from these changes (about one-fourth or one-third of the initial value at 40 min) was comparable to the observed decrease in $I_{\text{cat}}$ density during the decay (see, e.g., 1ATP in Fig. 2B). In contrast, only slight changes occurred in $V_{1/2}$ and $G_{\text{max}}$ when 10 mM ATP was present in the pipette (10ATP; closed symbols in Fig. 7C). These results strongly indicate that the main part of $I_{\text{cat}}$ decay can be accounted for by altered voltage-dependent gating and reduced maximal conductance of the channels underlying $I_{\text{cat}}$.

$I_{\text{cat}}$ Activated Bypassing the Muscarinic Receptor Shows Similar Characteristics

Finally, we investigated how the time course of $I_{\text{cat}}$ decay would be affected, if the current is activated bypassing the receptor. This was achieved by internal perfusion of 100 μM GTPγS to activate the G proteins (5, 42). As demonstrated in Fig. 8A and summarized in Fig. 8B, the features of GTPγS-induced $I_{\text{cat}}$ decay were essentially the same as those of $I_{\text{cat}}$ evoked via the muscarinic receptor; the magnitude of $I_{\text{cat}}$ stayed almost constant after reaching a maximum level, when 2...
mM ATP was included in the pipette together with Mg\(^{2+}\). In contrast, this effect could not be mimicked by AMP-PNP and was strongly attenuated by inclusion of alkaline phosphatase (Fig. 8B). Elimination of Mg\(^{2+}\) from the pipette also resulted in the inability of ATP to maintain GTP\(_{\gamma}S\)-induced \(I_{\text{cat}}\). There is, however, one notable difference compared with \(I_{\text{cat}}\) evoked by CCh. Two millimolar ATP (plus Mg\(^{2+}\)), which was not enough to completely prevent the time-dependent decay of \(I_{\text{cat}}\) evoked by CCh, was sufficient to virtually abolish the GTP\(_{\gamma}S\)-induced \(I_{\text{cat}}\) decay. This difference could be explained in part by the absence of altered receptor sensitivity or desensitization in GTP\(_{\gamma}S\)-induced \(I_{\text{cat}}\) (21).

**DISCUSSION**

The present study has clearly indicated that, in addition to \(R_a\), the perfusates and the protocol of receptor stimulation critically affect the behavior of \(I_{\text{cat}}\) when recorded via a single patch electrode. This is supported by the findings that 1) the effects of internally perfused ATP and GTP on \(I_{\text{cat}}\) occurred with a considerably slower time course than those of Cs\(^+\) loading on voltage-dependent Ca\(^{2+}\) current or Ca\(^{2+}\) chelation on Ca\(^{2+}\)-dependent K\(^+\) current, despite a similar rate of aqueous diffusion (time constant: 1–2 min) for ATP, GTP, Cs\(^+\), and BAPTA [a cell volume of 4.1 pl (13) and \(R_a\) of 10.7 MΩ were assumed; refer to the equation in Ref. 30]; and 2) the effects of internally applied ATP or GTP on \(I_{\text{cat}}\) became evident only after repeated receptor stimulation and were not recognizable at the first application of CCh (Figs. 1, 2, and 5A), while internal perfusion of Cs\(^+\) and Ca\(^{2+}\) buffers were effective. One possible explanation for such slowly appearing and stimulation-dependent effects of ATP and GTP (and presumably other nucleotides too) would be an extremely slow equilibration between the patch pipette and the subsarcolemmal space underneath \(I_{\text{cat}}\) channels. This might be due to the presence of intervening ATPases, kinases, high-affinity binding proteins (e.g., actin filaments), and intracellular pools sequestering nucleotide phosphates (6). Endogenous nucleotide

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**Fig. 6.** \(I_{\text{cat}}\) decay only modestly affects the receptor sensitivity. A: at a holding potential of −60 mV, various concentrations of CCh (0.1–100 \(\mu\)M) were sequentially puffed onto the cell. Traces at 20, 30, and 50 min after the onset of internal perfusion with 2ATP are superimposed. B: relationships between CCh concentration and \(I_{\text{cat}}\) amplitude at every 10 min after the onset of whole cell configuration. For better comparison, responses are normalized to those with 100 \(\mu\)M CCh for each cell tested (\(n = 5\)). Symbols and vertical bars indicate means ± SE.
phosphates tightly bound to intracellular proteins and vigorously produced in the vicinity of the \textit{I}_{\text{cat}} channel might account for the requirement of repeated receptor stimulation to uncover the effects of exogenously applied nucleotide phosphates. Whatever mechanisms are involved, it seems reasonable to say that data based on a single agonist application after a short internal perfusion time should be interpreted with great caution, since they may not faithfully reflect the real effects of internally applied nucleotide phosphates.

There are indeed some essential differences found in the effects of nucleotide phosphates between our results and those of a similar study in the same preparation where only the first response to CCh 5 min after internal perfusion was evaluated (1). According to this study 1) the stimulatory effect of internally applied high-energy phosphates on \textit{I}_{\text{cat}} was exclusively specific for phosphocreatine (ATP was almost ineffective) and did not require Mg\textsuperscript{2+} (but creatine together with a few millimolar ATP and Mg\textsuperscript{2+} is effective); 2) internal perfusion of GTP or total elimination of nucleotide phosphates and Mg\textsuperscript{2+} from the patch pipette did not affect \textit{I}_{\text{cat}} activity; 3) the inhibitory effect of ATP seemed inconsistent and the involvement of hydrolysis and the need of Mg\textsuperscript{2+} equivocal. These results are not easily interpretable using the standard knowledge about ATP, GTP, and other high energy phosphates. In contrast, our present data clearly indicate that ATP exerts (and also phosphocreatine; Ref. 40) a stimula-


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**ATP HYDROLYSIS ON MUSCARINIC CATION CHANNELS**

**Fig. 8.** GTPγS-induced \( I_{\text{cat}} \) shows similar dependence on ATP. \( V_H = -60 \) mV. Bath: PSS. A: time-dependent development of \( I_{\text{cat}} \) during internal perfusion of 100 \( \mu \)M GTPγS via a patch pipette with 2 mM ATP (a; 2ATP), 2 mM AMP-PNP (b; 2AMP-PNP), and 2 mM ATP + alkaline phosphatase (100 \( \mu \)g/ml; c; 2ATP + AP). Mg\(^{2+} \) (2.5 mM) was present in the pipette (see Table 1). B: time course of GTPγS-induced \( I_{\text{cat}} \) under various pipette conditions such as shown in A. Symbols and vertical bars indicate means ± SE from 5–9 cells. *Statistically significant differences at 30 min for 2ATP as a control* (\( P < 0.05 \) with Dunnett’s test).

The pharmacology of kinase and phosphatase inhibitors (Fig. 3D) suggests that the main part of the maintaining effect of MgATP on \( I_{\text{cat}} \) may be mediated by phosphorylation of tyrosine residues tightly associated with \( I_{\text{cat}} \) activity (16). Protein kinases A, G, or C, and calmodulin kinase II are unlikely to be involved in this (Fig. 3D), although they are frequently found as effective modulators of ion channels and receptors coupling to them (10, 21, 24). The type of tyrosine kinase/phosphatase responsible for maintaining \( I_{\text{cat}} \) activity remains elusive. However, one plausible candidate is the nonreceptor type tyrosine kinase Src, which is abundantly expressed in various types of smooth muscle and is thought to play a pivotal role in regulating the Ca\(^{2+} \) sensitivity of the contractile machinery (7, 32). Interestingly, Src has been implicated in the G protein/protein kinase C-mediated regulation of \( N \)-methyl-D-aspartate cation channels by muscarinic and lysophosphatidic acids in some neuronal tissues, where coimmunoprecipitation of the channel protein and Src has also been demonstrated (27, 36). It is also interesting to note that a similar dependence on intracellular MgATP is reported for the cardiac and islet \( \beta \)-cell ATP-sensitive K\(^+ \) channels as well as L-type voltage-dependent Ca\(^{2+} \) channels in cardiac and smooth muscle cells (see Introduction), both of which exhibit a time-dependent decline without MgATP that can preferentially be reversed by glycolytic production of ATP (26, 39). Because these properties appear to be shared by \( I_{\text{cat}} \) channels (40), it may be profitable to investigate the roles of cytoskeletal elements and phosphatidylinosities on \( I_{\text{cat}} \) activity, both of which are known to alter the rate of rundown or the availability of ATP-sensitive K\(^+ \) and voltage-dependent Ca\(^{2+} \) channels (11, 31).

Comparison of whole cell data with different free ATP concentration values has suggested that the free form of ATP may inhibit \( I_{\text{cat}} \) (2ATP vs. 10ATP/2Mg in Fig. 3). This was further corroborated by the experiments using flash photolysis of caged ATP (Fig. 4). The degree of ATP-mediated \( I_{\text{cat}} \) inhibition was not significantly affected when the current was activated by internally applied GTPγS in the presence of various protein kinase inhibitors. This suggests that enzymatic reactions may not be involved in the inhibitory effect of ATP and that its target site is located downstream of the muscarinic receptor, presumably on the \( I_{\text{cat}} \) channel protein. However, the observed time course of \( I_{\text{cat}} \) inhibition by photolytically released ATP seems rather slow (time constant \( \sim 10 \) s) when considering direct actions. One likely explanation is limited diffusion of ATP through the mechanisms discussed above, which may be valid even in the close proximity of the \( I_{\text{cat}} \) channel. Thus the most likely mechanism involved in the inhibitory effect of ATP is direct inhibition of \( I_{\text{cat}} \) channels, as has been suggested for the ATP-sensitive K\(^+ \) channels and Ca\(^{2+} \)-activated nonselective cation channels (31, 33, 34). However, more robust evidence should be provided under experimentally simpler conditions (e.g., single channel recordings).

The dependence of \( I_{\text{cat}} \) on intracellular GTP concentration has been investigated in some detail previously (42, 43). In these studies, the concentration of activated G...
protein, which is subject to the intensity of receptor stimulation or the extent of desensitization, has been implicated as a critical determinant of voltage-dependent gating and maximal conductance of $I_{cat}$ channels. We quantified more strictly the enhancing effect of GTP on $I_{cat}$ under the conditions in which [MgATP], [ATP], [Mg$^{2+}$], and [Ca$^{2+}$] were kept almost constant, and we found that activation of the $I_{cat}$ channel by GTP occurred most noticeably in its hundred micromolar range (apparent $K_d$ = $\sim 100$ μM; Fig. 5B).

Kinetic changes induced by GTP (43) are unexpectedly very similar to those observed for MgATP in the present study (Fig. 7). Both nucleotide phosphates cause a negative shift in the activation curve and an increase in the maximal conductance of $I_{cat}$ (Fig. 7) (43). Nevertheless, the effects of GTP and ATP are clearly distinguishable. GTP itself failed to slow the decay of $I_{cat}$ (Fig. 5A) but increased $I_{cat}$ density after a sufficiently long period of internal perfusion, if millimolar concentrations of MgATP were present (Fig. 5B). In contrast, ATP dose-dependently slowed $I_{cat}$ decay (Fig. 2B) but was not able to enhance steady-state $I_{cat}$ density if the concentration of coexisting GTP was low (Fig. 5B). It is thus likely that the main effect of GTP is to determine the extent of activation of $I_{cat}$ channels by changing the activated concentration of a G protein during receptor stimulation (42), whereas that of MgATP is to prime $I_{cat}$ channels for opening, presumably through a phosphorylation-dependent but G protein-independent mechanism. Such effects of ATP are highly analogous to those for the ATP-sensitive K$^+$ channels (8) and thus will deserve further investigation at a single-channel level.

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REFERENCES


