Journal of Physiology (1993), **470**, *pp.* 55–72
With 8 figures
Printed in Great Britain

ATP MODULATION OF CALCIUM CHANNELS IN CHROMAFFIN CELLS

BY L. GANDÍA*, A. G. GARCÍA† AND M. MORAD‡

From the Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085, USA

(*Received 5 May 1993*)

SUMMARY

1. The effects of externally applied micromolar concentrations of adenosine 5′-triphosphate (ATP) on Ca^{2+} currents (I_{Ca}) were studied in whole-cell clamped adrenaline-secreting chromaffin cells.

2. Ca^{2+} currents in chromaffin cells activated at about −40 mV, reached a maximum at 0 mV and had an apparent reversal potential at +50 to +60 mV, indicating the existence of only high voltage-activated Ca^{2+} channels.

3. ATP blocked Ca^{2+} current rapidly, reversibly and in a concentration-dependent manner (10^{-9}–10^{-4} M).

4. ATP did not completely block Ca^{2+} current even at the highest concentrations used (100 μM). The remaining component of Ca^{2+} current was characterized by slower activation and inactivation kinetics.

5. ATP blocked I_{Ca} even in the presence of nisoldipine and/or ω-conotoxin GVIA, suggesting that its modulatory role is not specific for L- and/or N-type Ca^{2+} channels.

6. Other adenine nucleotides also blocked the Ca^{2+} current partially. The order of potencies was ATP ⩾ ADP > AMP ⩾ adenosine, indicating that the ATP effects are most probably mediated by a P_2-type purinergic receptor.

7. Dialysis of the cells with an intracellular solution containing 1 mM guanosine 5′-*O*-thiodiphosphate (GDP-β-S) or pre-incubation of the cells with pertussis toxin (PTX) blocked the inhibitory effects of ATP.

8. Intracellular application of the non-hydrolysable GTP analogue guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S; 50 μM) also decreased I_{Ca} in a manner similar to that seen for ATP and significantly reduced the ATP inhibitory effect.

9. Conditioning pulses to potentials positive to +80 mV partly reversed the inhibitory effects of ATP on the Ca^{2+} current. The prepulse-induced enhancement of I_{Ca} depended on $[GTP]_i$-related G protein activity such that concentrations larger than 200 μM GTP, or GTP-γ-S (50 μM) were required for significant prepulse potentiation of the Ca^{2+} current, while dialysis with GDP-β-S prevented it.

* Present address: Departamento de Fisiología y Farmacología, Universidad de Salamanca, Spain.

† Present address: Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Spain.

‡ To whom correspondence should be addressed.

MS 1340

10. We conclude that the ATP, co-released with catecholamines in the intact adrenal gland, may inhibit the secretory process by down-regulating the Ca^{2+} channel via a P_2-type purinergic receptor coupled to a PTX-sensitive G protein.

INTRODUCTION

Extracellular application of adenosine 5′-triphosphate (ATP) at micromolar concentrations has been shown to regulate ionic channels and biological signalling in a wide variety of normal and transformed cell types. These biological responses seem to be mediated by different receptors, identified as P_2-purinergic receptors (Burnstock, 1990; Dubyak, 1991). Based on their selectivities for different nucleotides, at least three classes of ATP receptors have been identified (Burnstock, 1990) and correlated with, at least, three signal transduction pathways (Dubyak, 1991): (1) activation of ligand-gated cation channels; (2) activation of modulatory G proteins; and (3) formation of non-selective pores permeable to ions and small metabolites.

In chromaffin cells, ATP is co-stored (Winkler & Westhead, 1980) and co-released with catecholamines (in cat: Douglas, Poisner & Rubin, 1965; Rubin & Jaanus, 1967; in bovine: Stevens, Robinson, Van Dike & Stitzel, 1975; Castillo, Moro, del Valle, Sillero, García & Sillero, 1992). Since ATP causes inhibition (Chern, Herrera, Kao & Westhead, 1987) or facilitation (Chern, Kim, Slakey & Westhead, 1988; Kim & Westhead, 1989) of catecholamine release from isolated bovine chromaffin cells, it has been suggested that ATP and adenosine receptors might exert a modulatory role on secretion. These effects could be mediated through modulatory actions of these nucleotides on the high-threshold Ca^{2+} channels. Direct evidence for the regulatory role of ATP on the Ca^{2+} channels (I_{Ca}) has been recently presented by Diverse-Pierluissi, Dunlap & Westhead (1991), who found that ATP can induce both inhibition and enhancement of Ca^{2+} current through a PTX-sensitive pathway. Yet, in another series of experiments in phaeochromocytoma (PC12) cell line, ATP has been shown to regulate catecholamine secretion by activation of a non-specific cation channel carrying a maintained current (Nakazawa, Fujimori, Takanaka & Inoue, 1990*a*; Nakazawa, Inoue, Fujimori & Takanaka, 1990*b*; Inoue & Nakazawa, 1992).

Here we report on the effects of extracellular ATP on adrenaline-containing bovine chromaffin cells and examine the interaction of GTP analogues and I_{Ca}-facilitating conditioning pulses on the regulatory role of ATP on the Ca^{2+} channel. Our results showed that ATP rapidly and reversibly suppressed the Ca^{2+} channel via the activation of a PTX-sensitive regulatory G protein and that I_{Ca}-facilitating conditioning pulses lead to reversal of inhibition of ATP-suppressed I_{Ca}. Our results provide strong support for a direct role of G proteins in modulation of Ca^{2+} current by ATP and facilitating depolarizing prepulses. A preliminary report of this work has already appeared (Gandía & Morad, 1992).

METHODS

Preparation of chromaffin cells

Fresh adrenal glands were obtained from young calves at a local slaughterhouse. Adrenal medullary chromaffin cells were isolated from the adrenal medullae as previously described (Gandía, Casado, López & García, 1991) but with the following modifications. After the Percoll

gradient step, two bands of chromaffin cells were taken, washed once with Ca^{2+}–Mg^{2+}-free Locke buffer containing (mM): 154 NaCl, 5·6 KCl, 3·5 $NaHCO_3$, 11 glucose, 10 Hepes–NaOH, pH 7·2) and resuspended in 15 ml of Urografin 15% (Schering A. G., Germany). Ten millilitres of Urografin 7·5% were then added slowly to prevent the mixture of both solutions. Cells were centrifuged at 7500 r.p.m. for 20 min at 22 °C. This method allowed effective separation and purification of the noradrenaline- and adrenaline-containing chromaffin cells. Most of the experiments reported here were performed in cells obtained from the lower layer of the Percoll gradient, which consisted of over 90% of adrenaline-secreting cells (Moro, López, Gandía, Michelena & García, 1991), although similar results were obtained when cells from the upper layer of the Percoll gradient (containing more than 60% noradrenaline-secreting cells) were used. Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin, at a density of 25×10^3 cells/ml. Cells were plated on glass coverslips and maintained in an incubator under an atmosphere of air and 5% CO_2 mixture at 37 °C. Experiments were performed in cells from 2 to 8 days in culture. All experiments were done at room temperature (22–25 °C).

Measurement of Ca^{2+} currents

Ca^{2+} currents in chromaffin cells were recorded using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (22–25 °C) with a control Tyrode solution containing (mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 Hepes–NaOH, 10 glucose, 0·005 tetrodotoxin (TTX), pH 7·4.

In the experiments where Ca^{2+} channel blockers were used (Fig. 5), 10 mM Ba^{2+} (instead of 5 mM Ca^{2+}) was used as the charge carrier and a low-Na^+ and TEA-based solution (mM): 10 NaCl, 127 TEACl, 5 KCl, 1 $MgCl_2$, 10 $BaCl_2$, 10 Hepes–NaOH, 10 glucose, 0 TTX, pH 7·2 was used to minimize fast Na^+ currents. Except when indicated, cells were dialysed with a standard intracellular solution containing (mM): 10 NaCl, 100 CsCl, 20 TEACl, 5 MgATP, 14 EGTA, 0·2 GTP, 20 Hepes–CsOH, pH 7·2.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 3900 patch-clamp amplifier (Dagan Corporation, Minneapolis, MN, USA), allowing cancellation of capacitative transient and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pCLAMP software (Axon Instruments, Inc., Foster City, CA, USA) were used to acquire and analyse the data.

Solutions were exchanged using a multi-barrelled concentration-clamp device (Vibraspec, Inc., Philadelphia, PA, USA), the common outlet of which was placed within 100 μm of the cell to be patched.

RESULTS

Characterization of calcium currents in chromaffin cells

In primary cultures of adrenaline-secreting chromaffin cells, Ca^{2+} currents were recorded by 25 ms depolarizing pulses applied from a holding potential of −80 mV in 10 mV steps up to +60 mV. Other ionic currents were suppressed by dialysing the cells with the Cs^+-based intracellular solution and by bathing them in a Na^+-based solution containing TTX (5 μM). Ca^{2+} current activated slowly and showed little or no time-dependent inactivation on this time scale. Ca^{2+} currents elicited by depolarizing pulses from a holding potential of −80 mV activated at about −40 mV, peaked around 0 mV and showed an apparent reversal potential at about +50 to +60 mV. The peak Ca^{2+} current density in cells from different batches was 29·1 ± 2·2 pA/pF (mean ± S.E.M., $n = 38$) at 0 mV in 5 mM Ca^{2+}. Similar results were obtained when the cells were held at −100 mV, suggesting the existence of only high voltage-activated Ca^{2+} channels in chromaffin cells. These results agree with previous

reports (Fenwick, Marty & Neher, 1982; Artalejo, Dahmer, Perlman & Fox, 1991*a*), but contrast with the recent report of Diverse-Pierluissi *et al.* (1991) that describe the existence of a low-threshold Ca^{2+} channel in these cells.

Extracellular ATP suppressed Ca^{2+} currents

Figure 1 shows the effect of rapid and repetitive application of 1 μM ATP on Ca^{2+} current recorded in an adrenergic cell. The cell was held at −80 mV and 25 ms test

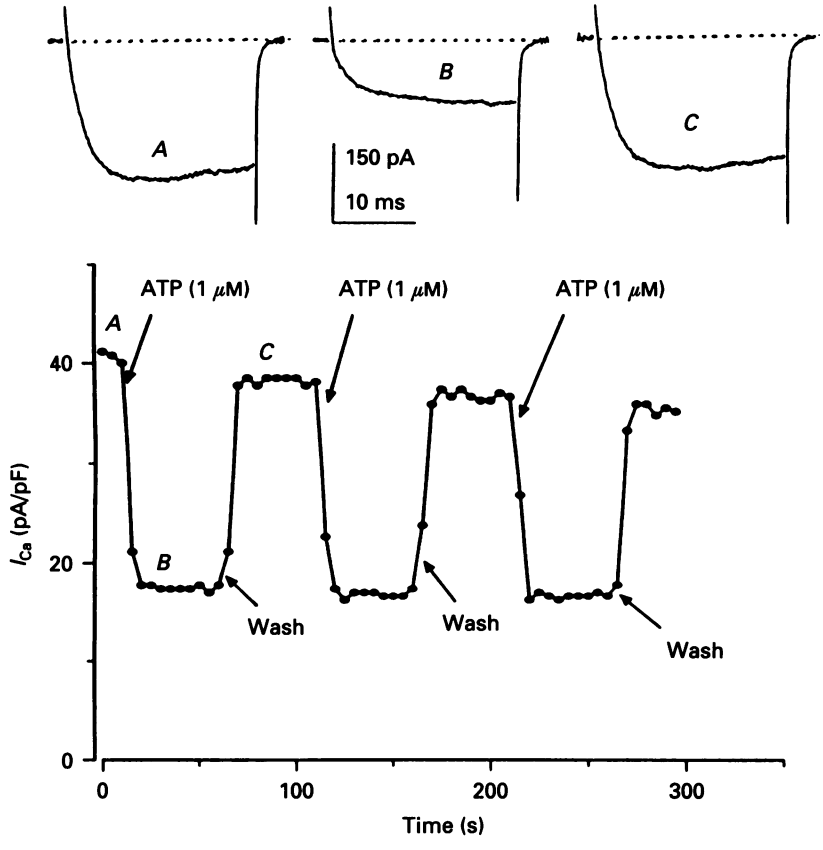

Fig. 1. Time course of inhibitory effects of 1 μM ATP on voltage-gated I_{Ca} in an adrenergic chromaffin cell (cell capacitance 12·93 pF). I_{Ca} was activated by 25 ms depolarizing pulses from −80 to 0 mV applied at a frequency of 0·2 Hz. As indicated by the arrows, ATP (1 μM) was added to or removed (Wash) from the perfusion solution. Solution exchanges were accomplished in less than 0·5 s by using a concentration clamp device. The inset at the top of the figure shows original current traces recorded at different times, before (*A*), during (*B*) and after washing out (*C*) ATP.

pulses to 0 mV were applied at 5 s intervals. Where indicated by arrows, ATP, at a final concentration of 1 μM, was applied or removed rapidly (< 100 ms) via a multi-barrelled concentration-clamp device (Sorbera & Morad, 1991). ATP rapidly ($\approx$ 5 s) and reversibly suppressed I_{Ca}. The effect of ATP remained stable for 2–3 min,

showing little or no desensitization. Comparison of the time course of Ca^{2+} current in the presence and absence of ATP (Fig. 1, inset) also suggests significant suppression in the kinetics of activation of I_{Ca} in the presence of ATP.

Figure 2 compares the voltage dependence of I_{Ca} in the absence and presence of 10 μM ATP in the extracellular solution. ATP (10 μM) significantly blocked I_{Ca} at all

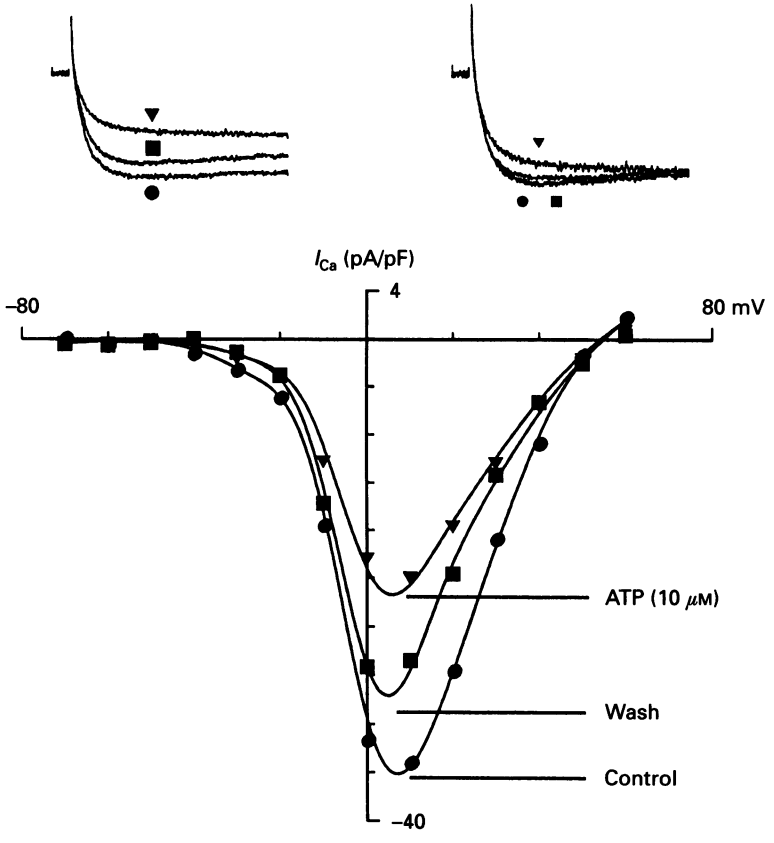

Fig. 2. Peak current–voltage relationship for I_{Ca} recorded in a noradrenergic chromaffin cell (cell capacitance, 10·15 pF) held at −80 mV activated by 25 ms depolarizing pulses applied at 0·2 Hz in 10 mV steps. The depolarizing protocol was applied at 5 min intervals, in the absence (Control) or the presence of ATP (10 μM) in the extracellular solution, and after a wash-out period of 5 min. Each concentration of ATP was superfused at least 2 min before the application of the depolarizing protocol. The inset shows the time course of I_{Ca} during a 25 ms test pulse to 0 mV and its modification by ATP. Right panel shows normalization of original current with respect to the final point.

potentials tested (Fig. 2). Comparison of the kinetics of activation and inactivation of I_{Ca} in the presence and absence of ATP, following normalization for the amplitude of the current, suggest slower gating kinetics in the presence of ATP (inset Fig. 2, see also Fig. 1). Ca^{2+} current recovered to within 85–90% of its original value following wash-out of ATP. Such decreases in Ca^{2+} current occurring within 10–15 min of cell dialysis were attributed to slow run-down of the Ca^{2+} current.

ATP inhibited I_{Ca} in a concentration-dependent manner at all potentials tested without altering significantly its voltage dependence. Figure 3 shows the dose dependence of the ATP effect on Ca^{2+} current. Data were fitted by a non-linear regression routine ($r^2 = 0{\cdot}996$), with a Hill coefficient of 1·92 and an apparent IC_{50} of

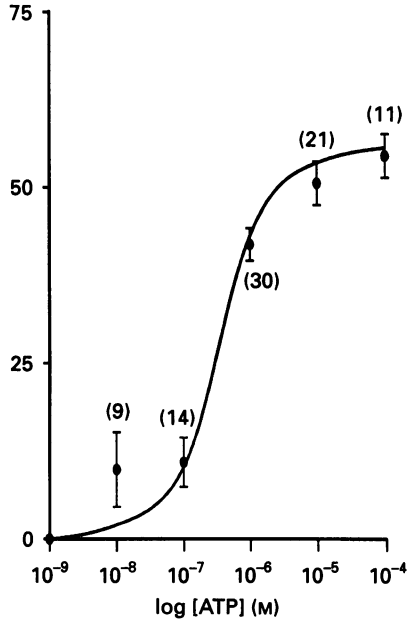

Fig. 3. Concentration–response relationship for the inhibitory effects of ATP on Ca^{2+} current in chromaffin cells. The residual blocking effect obtained in the presence of 10^{-9} M was considered to be due to run-down of the Ca^{2+} current and data have been normalized with respect to the current recorded in the presence of this concentration of ATP. Data are expressed as means ± S.E.M. for the number of cells indicated in parentheses.

0·56 μm. At the maximum concentration of ATP used (100 μM), only 60·2 ± 3·1 % of I_{Ca} was blocked, suggesting that Ca^{2+} current in chromaffin cells is carried by an ATP-sensitive and an ATP-insensitive population of Ca^{2+} channels. Consistent with this idea, recent reports suggest that bovine chromaffin cells may have more than one population of Ca^{2+} channels (Artalejo *et al.* 1991*a*; Artalejo, Mogul, Perlman & Fox, 1991*b*; Bossu, De Waard & Feltz, 1991*a*, *b*). Although the intracellular solution used minimized the run-down of the Ca^{2+} currents, the residual inhibition observed at the lower ATP concentrations (1 nM) could be caused by the run-down process.

Effects of ATP on the gating kinetics of I_{Ca}

The suppression of I_{Ca} by ATP was accompanied by slowing of the activation and inactivation kinetics of the Ca^{2+} current. This effect was more pronounced at the higher concentrations of ATP. In order to explore further this effect, two different protocols were used: (1) Ca^{2+} currents were activated by 25 ms test pulses to 0 mV from a holding potential of −80 mV in the absence and the presence of 10 μM ATP

(Fig. 4*A*). ATP decreased and significantly slowed the time course of its activation (see normalized I_{Ca}, Fig. 4*A*,*b*); (2) the inactivation kinetics of the Ca^{2+} current were studied using long (400 ms) depolarizing pulses to 0 mV from a holding potential of −110 mV. Figure 4*B* shows that while in control solutions, I_{Ca} partially inactivated

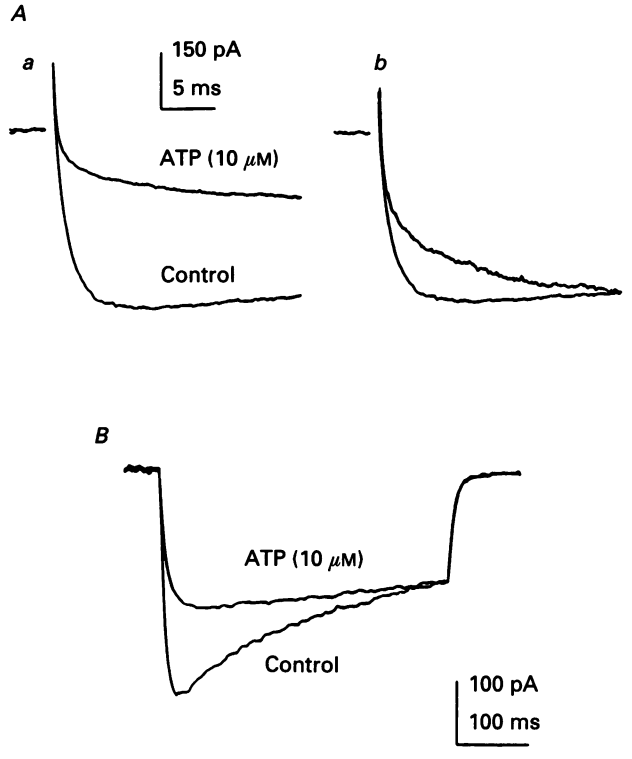

Fig. 4. Modifications in Ca^{2+} channel gating kinetics induced by superfusion with an ATP-containing solution. *A a*, original current traces recorded from an adrenergic cell when a 25 ms depolarizing pulse to 0 mV from a holding potential of −80 mV was applied in the absence (Control) or the presence of 10 μM ATP. *A b*, the current was normalized with respect to the final value in order to illustrate further how ATP slows the activation of the Ca^{2+} current. *B*, Ca^{2+} currents recorded during a 400 ms depolarizing pulse to 0 mV from a holding potential of −110 mV in the presence or the absence of ATP (10 μM).

within 400 ms, there was little or no inactivation during the same period in the presence of ATP. These results indicate that the ATP-insensitive component of the I_{Ca} has different activation and inactivation kinetics.

Effects of ATP in cells treated with Ca^{2+} channel blockers

In order to determine if the blocking effects of ATP were restricted to a given subtype of Ca^{2+} channel, the suppressive effect of ATP was examined in cells pretreated with Ca^{2+} channel blockers known to block either the L-type (nisoldipine) or N-type (ω-conotoxin GVIA) Ca^{2+} channels. Figure 5 shows that extracellular

application of ATP (10 μM) led to a 46±3% (n = 26) decrease in macroscopic Ba^{2+} currents. As in the experiments where Ca^{2+} (5 mM) was the charge carrier, this effect was fast and reversible upon removal of the nucleotide from the perfusate. In solutions containing nisoldipine (10–30 μM), where I_{Ba} was suppressed by 19±5% (n = 9), ATP continued to block an equivalent amount of the Ba^{2+} current (Fig. 5).

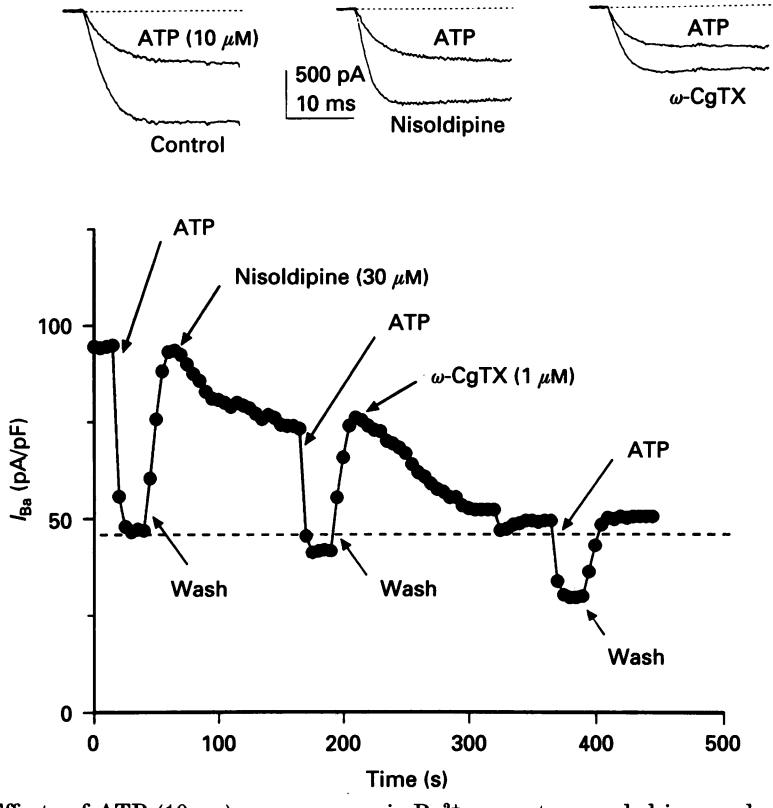

Fig. 5. Effects of ATP (10 μM) on macroscopic Ba^{2+} currents recorded in an adrenergic chromaffin cell (cell capacitance 13·74 pF) before and after the treatment with nisoldipine (30 μM) and ω-conotoxin (ω-CgTX; 1 μM). I_{Ba} was elicited by 25 ms depolarizing pulses to 0 mV from a holding potential of −80 mV applied at 5 s intervals. Top traces show linear leak- and capacitance-subtracted current traces recorded under the different perfusion conditions indicated in the figure.

When ω-conotoxin (1 μM) was used to block the N-type Ca^{2+} channels, Ba^{2+} current was inhibited by 46±4% (n = 10). In nisoldipine-treated cells, ω-conotoxin continued to block the current by 52±6% (n = 8). Removal of the toxin from the bathing solution did not result in recovery of the blocked current (Hans, Illes & Takeda, 1990; Artalejo, Perlman & Fox, 1992*a*). Figure 5 shows that ATP continued to block reversibly I_{Ba} even in ω-conotoxin-treated cells. ATP (10 μM) further blocked I_{Ba} in such cells by 31±3% (n = 14). Thus, it appears that ATP inhibits a population of Ca^{2+} channels other than N- or L-type Ca^{2+} channels.

Effects of other adenine nucleotides

ATP has been reported to interact with two types of purinergic receptors, P_1 and P_2 (Burnstock, 1990; Dubyak, 1991). Sensitivity of such receptors to different adenosine derivatives has served as a major tool for identification of the subtype of

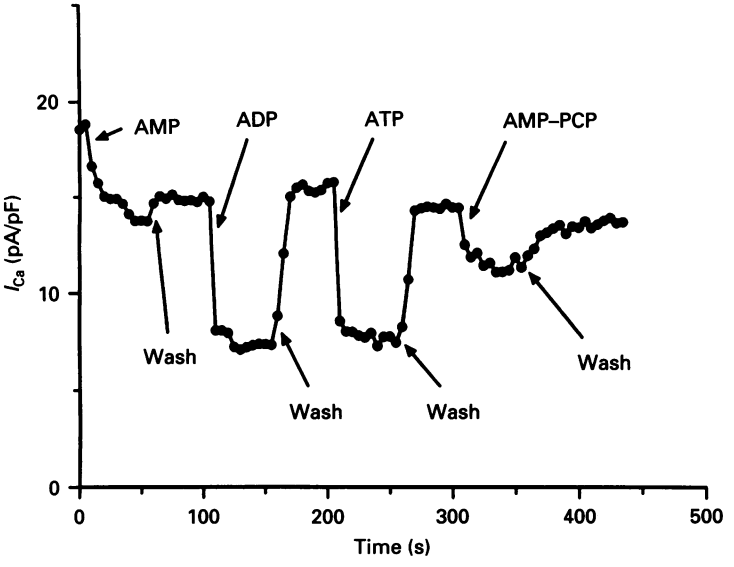

Fig. 6. Time course of Ca^{2+} current recorded in an adrenergic chromaffin cell (cell capacitance 10·42 pF) and its modification by different adenine nucleotides (AMP-PCP; β, γ-methylene ATP). Ca^{2+} current was activated by 25 ms depolarizing pulses to 0 mV from a holding potential of −80 mV applied at 5 s intervals. Arrows indicate the points where the nucleotides were added. All these compounds were superfused at a final concentration of 1 μM.

receptors. In order to identify the type of purinergic receptor mediating the inhibitory effect of ATP, we compared the effects of equimolar concentrations of adenosine, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), ATP, α, β-methylene-ATP (AMP-CPP) and β, γ-methylene-ATP (AMP-PCP) on the Ca^{2+} current. Figure 6 compares the effects of different nucleotides on I_{Ca} of an adrenergic chromaffin cell. All nucleotides were superfused at a final concentration of 1 μM. Results from this type of experiment showed that while 1 μM AMP had a small blocking effect on Ca^{2+} currents (17·8±2·7%, $n = 10$), 1 μM ADP was equivalent to ATP in blocking the current (48·2±3·4%, $n = 11$ *vs.* 47·8±4·2%, $n = 11$, respectively), a pattern quite similar to that observed by Diverse-Pierluissi *et al.* (1991). Thus, the order of Ca^{2+}-channel blocking potency of the nucleotides (ATP ⩾ ADP ≫ AMP > adenosine) appears to support the possibility that P_2-purinergic receptors mediate the regulatory effect of ATP on the Ca^{2+} channels.

When AMP-CPP or AMP-PCP (Fig. 6) were used to characterize further the subtype of P_2-receptor, a weaker Ca^{2+} current inhibitory effect was found compared

to ATP (23·6±5·8%, $n = 5$ and 22·2±2·1%, $n = 7$, for AMP-CPP and AMP-PCP respectively). Furthermore, no desensitization accompanied the blocking effect of any of the adenine nucleotides, suggesting that the receptor mediating the ATP effect of the Ca^{2+} channel is not likely to be of the P_{2x}-subtype.

The blocking effect of ATP is mediated by G proteins

To test whether the Ca^{2+} channel blocking effects of ATP were mediated by regulatory G proteins, cells were dialysed with non-hydrolysable analogues of

TABLE 1. Ca^{2+} current density and inhibition by ATP under different conditions known to modify the activity of regulatory G proteins

	Ca^{2+} current (pA/pF)	ATP (1 μM) (%)	ATP (10 μM) (%)
GTP	28·8±2·0 (38)	49·4±2·2 (30)	56·9±3·0 (21)
GTP-γ-S	21·2±4·9 (5)	—	4·4±4·7 (4)
GDP-β-S	34·4±5·2 (13)	8·8±2·5 (8)	10·9±3·8 (9)
PTX	22·8±3·1 (7)	14·9±6·5 (4)	16·1±5·5 (5)

Data are indicated as pA/pF and as percentage of inhibition with respect to the current recorded in absence of ATP and are expressed as means±S.E.M. of the number of cells indicated in parentheses. Concentrations of guanine nucleotides used were (mM): 0·2 GTP, 0·05 GTP-γ-S, 1 GDP-β-S. PTX was used at a final concentration of 500 ng/ml.

guanine nucleotides at concentrations that fully activate (GTP-γ-S) or completely block (GDP-β-S) the turnover of the G proteins.

In chromaffin cells dialysed with intracellular solutions containing GDP-β-S (1 mM), ATP failed to show significant inhibitory effects on I_{Ca} (Fig. 7*A*). In these cells, 1 and 10 μM ATP blocked I_{Ca} by only 8·8±2·5 and 10·9±3·8% respectively compared to 49·4±2·2 and 56·9±3·0% (Table 1) in cells dialysed with no GDP-β-S.

In cells incubated for 4 h in solutions containing pertussis toxin (PTX, 500 ng/ml) to ribosylate the α-subunit of the G_i/G_o regulatory proteins, 1–10 μM ATP also failed to have significant inhibitory effect on I_{Ca} (Table 1). This finding is consistent with those of Fig. 7*A* and further suggests that a PTX-sensitive G_i/G_o subtype of regulatory G protein mediates the suppressive effects of ATP on I_{Ca}.

The upper panel of Fig. 7*B* shows that when a chromaffin cell was dialysed with 50 μM GTP-γ-S, I_{Ca} decreased during the 5–10 min of dialysis. Similar data were obtained in four other cells, where I_{Ca} decreased from 449±58 pA to 177±29 pA ($n = 5$, $P < 0·005$) at the end of the dialysis period. This decrease in I_{Ca} was accompanied by changes in the time constant of activation of I_{Ca} from 1·97±0·2 ms, at the onset of dialysis, to 3·02±0·34 ms following full GTP-γ-S dialysis ($n = 5$, $P < 0·05$). This finding suggests that in addition to inhibition of I_{Ca}, GTP-γ-S also modulated the gating kinetics of the Ca^{2+} channel. Exposure of these cells to ATP (10 μM) failed to block further the remaining Ca^{2+} current (Fig. 7*B*, lower panel). Similar results were obtained in three other cells, where the inhibitory effect of ATP on I_{Ca} was only 4·4±4·7% ($n = 4$) (see also Table 1).

Depolarizing prepulses reverse I_{Ca} inhibitory effects of ATP

Application of conditioning pulses to highly positive potentials appears to reverse the blocking effects of a variety of neurotransmitters on Ca^{2+} current (Swandulla, Carbone & Lux, 1991). We investigated the effect of such conditioning pulses on the

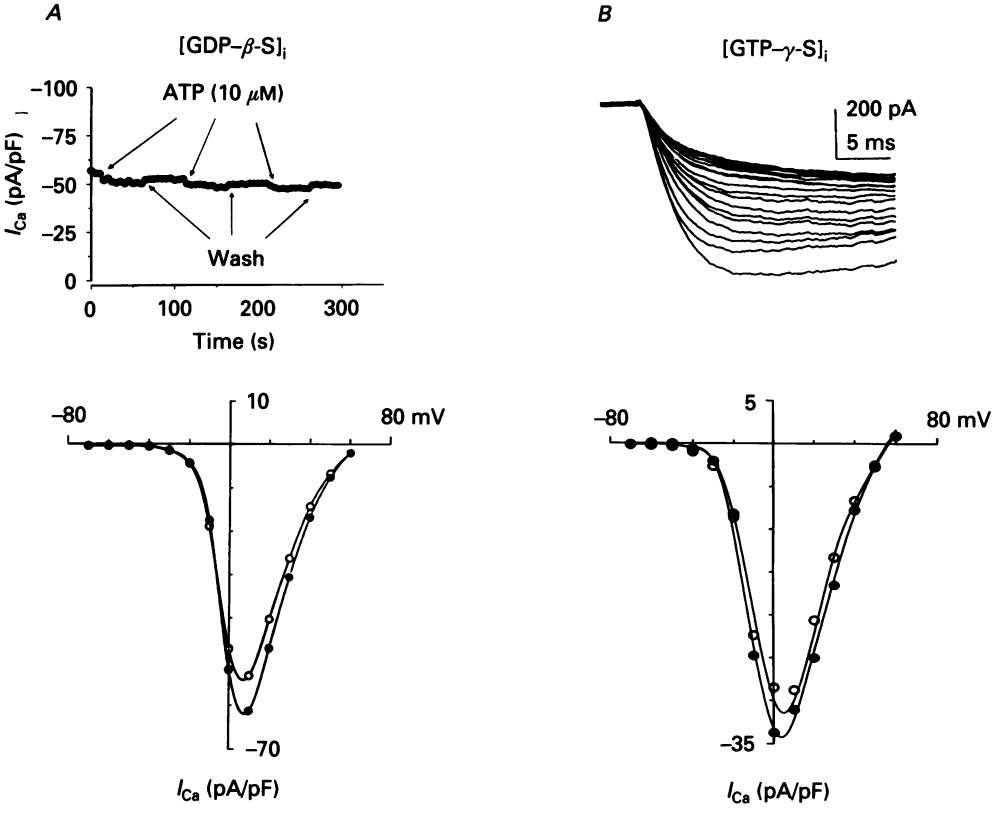

Fig. 7. Modulation by GTP analogues of ATP inhibitory effects. *A*, upper panel shows the time course for the suppressive effects of 10 μM ATP on I_{Ca} recorded in an adrenergic cell dialysed with an intracellular solution containing 1 mM GDP-β-S. After a dialysing period of 10 min, Ca^{2+} currents were studied by application of 25 ms depolarizing pulses to +10 mV from a holding potential of −80 mV applied at 5 s intervals. Arrows indicate when ATP was added to or removed from the extracellular solution. Lower panel shows the voltage dependence for I_{Ca} recorded in the absence (●) or the presence (○) of 10 μM ATP. *B*, upper panel shows superimposed leak- and capacitance-subtracted current traces recorded during a 10 min dialysing period with an intracellular solution containing the non-hydrolysable GTP analogue GTP-γ-S (50 μM). Lower panel shows the voltage dependence of I_{Ca} in the same cell recorded after the dialysing period in the absence (●) and the presence (○) of ATP (10 μM).

inhibitory effects of ATP on I_{Ca}. In these experiments, Ca^{2+} current was first activated by a 15–200 ms depolarizing pulse to 0 mV from a holding potential of −110 mV. After 30 s, the procedure was repeated but this time 35 ms prior to the

pulse a 25 ms conditioning clamp pulse to +100 mV was applied (see protocol in inset of Fig. 8). The upper panel of Fig. 8 shows that when the prepulse was applied to a cell dialysed with the standard intracellular solution, only a small increase in I_{Ca} was observed (15±2%, $n = 35$). These results are quantitatively similar to those

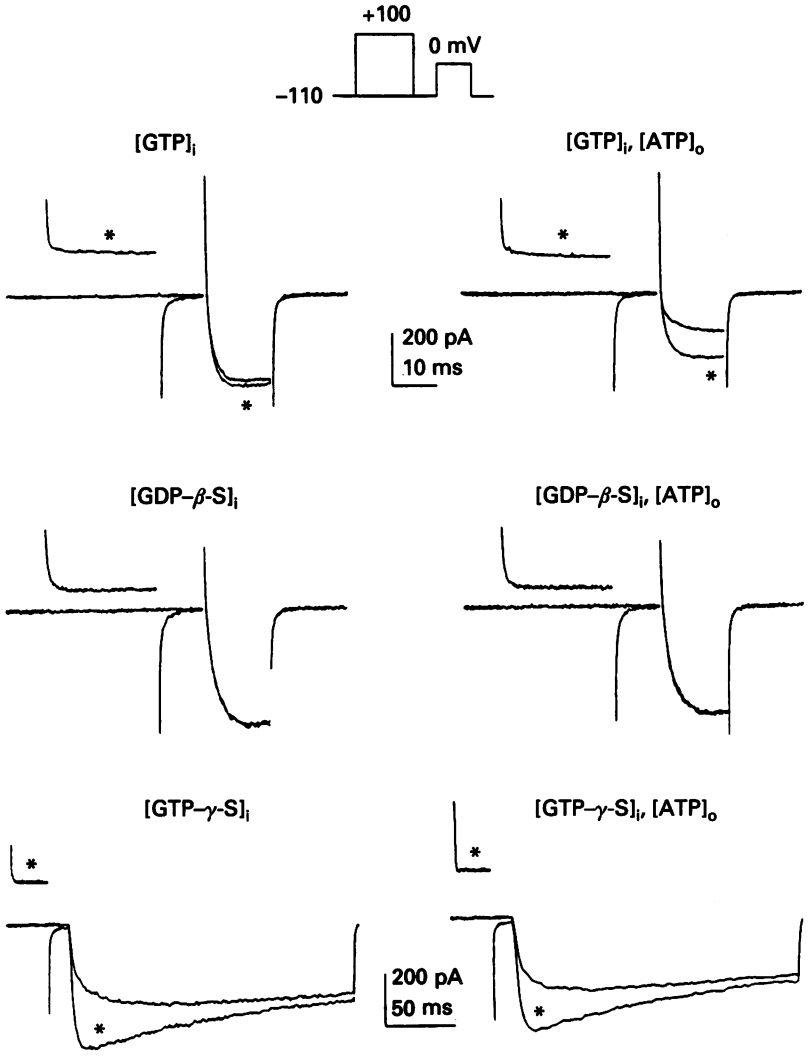

Fig. 8. Effects of the application of conditioning prepulses under different dialysing conditions. Ca^{2+} current was activated by a 15 ([GTP]$_i$ and [GDP-β-S]$_i$) or 200 ms ([GTP-γ-S]$_i$) depolarizing step to 0 mV from a holding potential of −110 mV (inset). After 30 s, the procedure was repeated but a 25 ms conditioning clamp pulse to +100 mV was applied 35 ms before the pulse. Left panels show the effect of prepulse application under the dialysing conditions indicated. In the right panels, an identical protocol was applied but in the presence of 1–10 μM ATP in the external solution.

previously obtained by Fenwick *et al.* (1982) and Hoshi, Rothlein & Smith (1984), but different from those recently reported by Artalejo *et al.* (1991*a*). On the other hand, when I_{Ca} had been first blocked by application of 10 μM ATP (right upper

panel), the prepulse induced a 95±12% ($n = 15$) increase in I_{Ca}. In cells dialysed with 1 mM GDP-β-S, no significant modifications of I_{Ca} occurred either with the depolarizing prepulse procedure, or with the application of extracellular ATP (Fig. 8, middle panels). On the other hand, in cells dialysed with GTP-γ-S (50 μM), application of the prepulse led to an increase of I_{Ca} and enhancement of its activation

TABLE 2. Effect of depolarizing prepulses applied under different intracellular and/or extracellular solutions

	Control	ATP (10 μM)
0 GTP, 5 MgATP External 5 Ca	113±1 (5)	155±15 (5)*
0·2 GTP, 5 MgATP External 5 Ca	115±2 (35)	195±12 (15)**
0·2 GTP, 5 MgATP External 10 Ba	116±4 (12)	165±7 (10)**
0 GTP, 2 MgATP External 10 Ba	108±2 (15)	163±6 (12)**
1 GDP-β-S, 5 MgATP External 5 Ca	107±2 (12)	109±3 (7) n.s.
0·05 GTP-γ-S, 5 MgATP External 5 Ca	202±29 (6)	165·9 (3) n.s.

Data are expressed as percentages with respect to the control current (100%) obtained in the absence of prepulse. All concentrations given are millimolar. Data are means±S.E.M. of the number of experiments shown in parentheses. Statistical differences using Student's t test were: n.s., non-significant; *$P < 0.05$ and **$P < 0.005$ with respect to the control in the absence of ATP.

and inactivation kinetics (Fig. 8, lower panel). Addition of ATP (10 μM) in such cells did not further alter the Ca^{2+} current either prior to or following the prepulse application. Table 2 summarizes the results obtained when depolarizing prepulses were applied under different conditions. These results show a significant facilitation of the Ca^{2+} current only when ATP was present or when the cells were dialysed with GTP-γ-S suggesting that the facilitation observed might be related to a process of voltage-dependent disinhibition of the Ca^{2+} channel that had been previously inhibited by the activation of a modulatory G protein.

DISCUSSION

The major finding of the present study is that micromolar concentrations of extracellular ATP inhibit the high-threshold Ca^{2+} channels in chromaffin cells by suppressing their gating kinetics. This effect appears to be mediated via a purinergic receptor coupled to G_i/G_o regulatory proteins. These findings provide support for the modulatory role of the extracellular ATP co-released with catecholamines in regulation of Ca^{2+} channels and secretion.

Ca^{2+} channel modulating effects of ATP

Our finding consistently showed that in chromaffin cells extracellular ATP, at micromolar concentrations, blocked Ca^{2+} current in a concentration-dependent manner (Figs 1, 2 and 5). Although these results are consistent with the data of

Diverse-Pierluissi *et al.* (1991) concerning the inhibitory effect of ATP on the high-threshold Ca^{2+} channels, they do not provide evidence for the enhancing effect of ATP reported by these authors. The reason for this discrepancy is not readily apparent.

ATP slowed the activation kinetics of the voltage-gated Ca^{2+} channel as it blocked the channel. A similar effect on the gating kinetics of the Ca^{2+} channel has been observed in several neurons and neurotransmitters: for GABA (Grassi & Lux, 1989); adenosine (Kasai & Aosaki, 1989); noradrenaline (Aicardi, Pollo, Sher & Carbone, 1991); for a recent review of the topic see Swandulla *et al.* (1991). Although the reasons for the suppression of the activation kinetics of I_{Ca} are not as yet clear, two possible mechanisms have been proposed. (i) Suppressions may result from the preferential block of a transient N-type Ca^{2+} channel thus unmasking a more slowly activating current component of an L-type variety (Lipscombe, Kongsamut & Tsien, 1989). This is, however, not a likely possibility since in ω-conotoxin-treated cells (where N-type Ca^{2+} channels are blocked) 10 μM ATP continued to block the current (Fig. 5). (ii) The slow activation of the remaining current might correspond to the voltage-dependent unblocking of the Ca^{2+} channel (Bean, 1989; Grassi & Lux, 1989; Kasai & Aosaki, 1989; López & Brown, 1991).

ATP has also been reported to activate a non-selective cation channel in chromaffin-derived PC12 cells (Nakazawa *et al.* 1990*a*, *b*; Inoue & Nakazawa, 1992). Although ATP is known to activate non-selective cation channels in other cell types (Bean, 1992), we have failed to activate such a current using concentrations of ATP ranging from 1 nM to 100 μM in over 100 cells tested. The failure to activate an ATP-gated non-selective cation channel could not have arisen from possible desensitization of ATP binding site as the speed of change of solutions ranged from 15 to 150 ms, a value significantly faster than that used by others to activate the channel. Possible ATP-induced increases in Ca^{2+} permeability of the membrane under such conditions, could be argued to cause the suppression of Ca^{2+} channel through a Ca^{2+}-induced inactivation mechanism (Eckert & Chad, 1984). We do not believe this is a likely possibility in that: (1) we failed to activate any non-selective cation channel; (2) in cells where Ba^{2+} was used as the charge carrier through the channel ATP continued to suppress I_{Ca} to the same extent as that seen in Ca^{2+}-containing solutions (Fig. 5); (3) chromaffin cells were dialysed with 10–14 mM EGTA, making a significant rise in intracellular calcium highly improbable.

The order of potency of different adenine nucleotides (ATP $\geqslant$ ADP $\gg$ AMP $>$ adenosine) in suppressing I_{Ca} indicates that the inhibitory effects of ATP are likely to be mediated by a P_2-type purinergic receptor. Recently the presence of a P_{2y}-receptor has been reported in chromaffin cells (Pintor, Torres, Castro & Miras-Portugal, 1991). Although no specific ligands for P_{2y}-receptors were used in the present work, the finding that the ATP analogues AMP-CPP or AMP-PCP had little effect on I_{Ca} makes it unlikely that a P_{2x}-purinergic receptor mediates the inhibitory effects of ATP on the Ca^{2+} currents. P_{2y}-receptors seem to stimulate the inositol phospholipid metabolism through a G protein-modulated pathway (Dubyak, 1991), that would lead to an increase in calcium release from intracellular stores.

G protein regulation of ATP effect

Our results suggest that a PTX-sensitive regulatory G protein modulates the inhibitory effects of ATP on Ca^{2+} currents (Figs 7 and 8). Intracellular applications of GTP-γ-S have been already shown to mimic the blocking effects of different neurotransmitters on neuronal calcium currents: for noradrenaline (Holz, Rane & Dunlap, 1986; Aicardi *et al.* 1991); GABA (Scott & Dolphin, 1986; Grassi & Lux, 1989); opioid peptides (Hescheler, Rosenthal, Trautwein & Schultz, 1987); adenosine (Kasai & Aosaki, 1989). It has been suggested (Swandulla *et al.* 1991) that the inhibition of Ca^{2+} channel induced by either neurotransmitters or intracellular GTP can be relieved with strong depolarizations which may reorganize the negatively charged groups of the G proteins leading to the reversal of inhibition of the channel. In support of this idea, we consistently found that a conditioning depolarizing pulse to positive potentials did not significantly increase I_{Ca} when the activity of G proteins was in a basal (control) or inhibited (dialysis with GDP-β-S) state. On the other hand, under conditions that partially or fully activated G proteins, such as extracellular application of ATP or intracellular dialysis with GTP-γ-S, conditioning pulses not only reversed the inhibition of I_{Ca} but also recovered the faster activation and inactivation kinetics of the channel (Fig. 8). Our data are thus consistent with the general modulatory role of G proteins in regulation of Ca^{2+} channel by neurotransmitters. Facilitation of Ca^{2+} currents in chromaffin cells has been previously described (Fenwick *et al.* 1982; Hoshi *et al.* 1984; Hoshi & Smith, 1987; Artalejo *et al.* 1991*a*), and related to the activation of a single class of Ca^{2+} channels (Hoshi *et al.* 1984; Hoshi & Smith, 1987) or to the recruitment of a different type of Ca^{2+} channel. Our data, suggesting the existence of a voltage-dependent reversal of inhibition process that allows the recovery of the neurotransmitter-suppressed current seems to be quite different from the facilitation of the Ca^{2+} current described by Artalejo *et al.* (1991*a*) induced by strong or repetitive depolarizations in chromaffin cells, a process that has been recently postulated to be independent of G protein modulation (Artalejo, Rossie, Perlman & Fox, 1992*b*).

A regulatory role for G proteins in secretory activity of chromaffin cells has already been postulated by Ceña, Brocklehurst, Pollard & Rojas (1991) who have reported increased catecholamine secretion in PTX-treated cells resulting from the removal of the tonic inhibition of G_i/G_o subset of regulatory proteins on the Ca^{2+} channels. What kind of inhibitory G protein mediates the suppressive effects of ATP on I_{Ca} is not as yet clear. Most likely, a G_o rather than G_i protein regulates the Ca^{2+} channel in chromaffin cells, since a G_o-type protein appears to mediate the transmitter-induced inhibition of neuronal Ca^{2+} channels not phosphorylated by cyclic AMP (Scott & Dolphin, 1986; Holz *et al.* 1986; Kleus, Hescheler, Ewel, Rosenthal, Schutz & Witting, 1991).

Functional implications of ATP effects

Questions regarding a possible functional role of the various compounds co-stored in the chromaffin granules and co-released with catecholamines, have remained unanswered. We have recently examined whether some of these substances modulate the Ca^{2+} current of chromaffin cells. Because the internal pH of secretory granules is about 5·6 (Johnson, 1987), it is probable that secretion is accompanied by a localized

change of $[H^+]_o$ which may inhibit Ca^{2+} channels. Consistent with such an idea, protons were found to block Ca^{2+} currents effectively (Callewaert, Johnson & Morad, 1991).

Some of the richest components of adrenergic secretory vesicles are the adenine nucleotides, of which ATP comprises 90% (Winkler & Westhead, 1980). It has already been shown that ATP is co-released with adrenaline and noradrenaline from the perfused intact bovine adrenal gland stimulated with acetylcholine or high K^+, in the presence of either Ca^{2+} or Ba^{2+} (Castillo *et al.* 1992). Our results indicate that extracellular ATP also down-regulates Ca^{2+} currents in chromaffin cells. Such modulatory effects seem to be mediated by a pertussis toxin-sensitive G_i/G_o protein, and might contribute to the regulation of catecholamine release. It is probable therefore that agents or factors co-stored and co-released during adrenergic secretion may serve as effective local inhibitors of secretion through modulation of the Ca^{2+} channel.

We thank Dr Lars Cleemann for critical reading of the manuscript. This study was supported by NIH grant HL 16152 and NATO grant CRG 900986. L. Gandía was supported by a fellowship from the Ministerio de Educación y Ciencia, Spain.

REFERENCES

AICARDI, G., POLLO, A., SHER, E. & CARBONE, E. (1991). Noradrenergic inhibition and voltage-dependent facilitation of ω-conotoxin-sensitive Ca channels in insulin-secreting RINm5F cells. *FEBS Letters* **281**, 201–204.

ARTALEJO, C. R., DAHMER, M. K., PERLMAN, R. L. & FOX, A. P. (1991*a*). Two types of Ca^{2+} currents are found in bovine chromaffin cells: facilitation is due to the recruitment of one type. *Journal of Physiology* **432**, 681–707.

ARTALEJO, C. R., MOGUL, D. J., PERLMAN, R. L. & FOX, A. P. (1991*b*). Three types of bovine chromaffin cell Ca^{2+} channels: facilitation increases the open probability of a 27 pS channel. *Journal of Physiology* **444**, 213–240.

ARTALEJO, C. R., PERLMAN, R. L. & FOX, A. P. (1992*a*). ω-Conotoxin GVIA blocks a Ca^{2+} current in chromaffin cells that is not of the "classic" N type. *Neuron* **8**, 85–95.

ARTALEJO, C. R., ROSSIE, S., PERLMAN, R. & FOX, A. P. (1992*b*). Voltage-dependent phosphorylation may recruit Ca^{2+} current facilitation in chromaffin cells. *Nature* **358**, 63–66.

BEAN, B. P. (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**, 153–156.

BEAN, B. P. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends in Pharmacological Sciences* **13**, 87–90.

BOSSU, J. L., DE WAARD, M. & FELTZ, A. (1991*a*). Inactivation characteristics reveal two calcium currents in adult bovine chromaffin cells. *Journal of Physiology* **437**, 603–620.

BOSSU, J. L., DE WAARD, M. & FELTZ, A. (1991*b*). Two types of calcium channels are expressed in adult bovine chromaffin cells. *Journal of Physiology* **437**, 621–634.

BURNSTOCK, G. (1990). Purinergic mechanisms. *Annals of the New York Academy of Sciences* **603**, 1–18.

CALLEWAERT, G., JOHNSON, R. G. & MORAD, M. (1991). Regulation of the secretory response in bovine chromaffin cells. *American Journal of Physiology* **260**, C851–860.

CASTILLO, C. J. F., MORO, M. A., DEL VALLE, M., SILLERO, A., GARCÍA, A. G. & SILLERO, M. A. G. (1992). Diadenosine tetraphosphate (AP_4A) is co-released with ATP and catecholamines from bovine adrenal medulla. *Journal of Neurochemistry* **59**, 723–732.

CEÑA, V., BROCKLEHURST, K. W., POLLARD, H. B. & ROJAS, E. (1991). Pertussis toxin stimulation of catecholamine release from adrenal medullary chromaffin cells: mechanism may be by direct activation of L-type and G-type calcium channels. *Journal of Membrane Biology* **122**, 23–31.

CHERN, Y. J., HERRERA, M., KAO, L. S. & WESTHEAD, E. (1987). Inhibition of catecholamine

secretion from bovine chromaffin cells by adenine nucleotides and adenosine. *Journal of Neurochemistry* **48**, 1573–1576.

CHERN, Y. J., KIM, K. T., SLAKEY, L. L. & WESTHEAD, E. (1988). Adenosine receptors activate adenylate cyclase and enhance secretion from bovine adrenal chromaffin cells in the presence of forskolin. *Journal of Neurochemistry* **50**, 1484–1493.

DIVERSE-PIERLUISSI, M., DUNLAP, K. & WESTHEAD, E. W. (1991). Multiple actions of extracellular ATP on calcium currents in cultured bovine chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **88**, 1261–1265.

DOUGLAS, W. W., POISNER, A. M. & RUBIN, R. P. (1965). Efflux of adenine nucleotides from perfused adrenal glands exposed to nicotine and other chromaffin cell stimulants. *Journal of Physiology* **179**, 130–137.

DUBYAK, G. R. (1991). Signal transduction by P_2-purinergic receptors for extracellular ATP. *American Journal of Respiratory Cell and Molecular Biology* **4**, 295–300.

ECKERT, R. & CHAD, J. E. (1984). Inactivation of calcium channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982). Sodium and calcium channels in bovine chromaffin cells. *Journal of Physiology* **331**, 599–635.

GANDÍA, L., CASADO, L. F., LOPEZ, M. G. & GARCÍA, A. G. (1991). Separation of two pathways for calcium entry into chromaffin cells. *British Journal of Pharmacology* **103**, 1073–1078.

GANDÍA, L. & MORAD, M. (1992). Adenosine 5-triphosphate (ATP) modulates the calcium channel in chromaffin cells. *Biophysical Journal* **61**, A 400.

GRASSI, F. & LUX, H. D. (1989). Voltage-dependent GABA-induced modulation of calcium currents in chick sensory neurons. *Neuroscience Letters* **105**, 113–119.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HANS, M., ILLES, P. & TAKEDA, K. (1990). The blocking effects of ω-conotoxin on Ca current in bovine chromaffin cells. *Neuroscience Letters* **114**, 63–68.

HESCHELER, J., ROSENTHAL, W., TRAUTWEIN, W. & SCHULTZ, G. (1987). The GTP-binding protein, G_o, regulates neuronal calcium channels. *Nature* **325**, 445–447.

HOLZ, G. G. IV, RANE, S. G. & DUNLAP, K. (1986). GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* **319**, 670–672.

HOSHI, T., ROTHLEIN, J. & SMITH, T. (1984). Facilitation of Ca^{2+} channel currents in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **81**, 5871–5875.

HOSHI, T. & SMITH, S. J. (1987). Large depolarization induces long openings of voltage-dependent calcium channels in adrenal chromaffin cells. *Journal of Neuroscience* **7**, 571–580.

INOUE, K. & NAKAZAWA, K. (1992). ATP receptor-operated Ca^{2+} influx and catecholamine release from neural cells. *News in Physiological Sciences* **7**, 56–59.

JOHNSON, R. G. (1987). Proton pumps and chemosmotic coupling as a generalized mechanism for neurotransmitter and hormone transport. *Annals of the New York Academy of Sciences* **493**, 162–177.

KASAI, H. & AOSAKI, T. (1989). Modulation of Ca-channel current by an adenosine analogue mediated by a GTP-binding protein in chick sensory neurons. *Pflügers Archiv* **414**, 145–149.

KIM, K. T. & WESTHEAD, E. (1989). Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **86**, 9881–9885.

KLEUSS, C., HESCHELER, J., EWEL, C., ROSENTHAL, W., SCHULTZ, G. & WITTING, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature* **353**, 43–48.

LIPSCOMBE, D., KONGSAMUT, S. & TSIEN, R. W. (1989). α-Adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. *Nature* **340**, 639–642.

LÓPEZ, H. S. & BROWN, A. M. (1991). Correlation between G protein activation and reblocking kinetics of Ca^{2+} channel currents in rat sensory neurons. *Neuron* **7**, 1061–1068.

MORO, M. A., LÓPEZ, M. G., GANDÍA, L., MICHELENA, P. & GARCÍA, A. G. (1991). Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. *Analytical Biochemistry* **185**, 243–248.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990*a*). An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *Journal of Physiology* **428**, 257–272.

NAKAZAWA, K., INOUE, K., FUJIMORI, K. & TAKANAKA, A. (1990*b*). ATP-activated single-channel currents recorded from cell-free patches of pheochromocytoma PC12 cells. *Neuroscience Letters* **119**, 5–8.

PINTOR, J., TORRES, M., CASTRO, E. & MIRAS-PORTUGAL, M. T. (1991). Characterization of diadenosine tetraphosphate (AP_4A) binding sites in cultured chromaffin cells: evidence for a P_{2y} site. *British Journal of Pharmacology* **103**, 1980–1984.

RUBIN, R. P. & JAANUS, S. D. (1967). The release of nucleotide from the adrenal medulla by indirectly acting sympathomimetic amines. *Biochemical Pharmacology* **16**, 1007–1012.

SCOTT, R. H. & DOLPHIN, A. D. (1986). Regulation of calcium currents by a GTP analogue: potentiation of (−)-baclofen-mediated inhibition. *Neuroscience Letters* **69**, 59–64.

SORBERA, L. A. & MORAD, M. (1991). Modulation of cardiac sodium channels by cAMP receptors on the myocyte surface. *Science* **253**, 1286–1289.

STEVENS, P., ROBINSON, R. L., VAN DIKE, K. & STITZEL, R. (1975). Synthesis, storage and drug-induced release of ATP-8-^{3}H in the perfused bovine adrenal gland. *Pharmacology* **13**, 40–55.

SWANDULLA, D., CARBONE, E. & LUX, H. D. (1991). Do calcium channel classifications account for neuronal calcium channel diversity? *Trends in Neurosciences* **14**, 46–51.

WINKLER, H. & WESTHEAD, E. (1980). The molecular organization of adrenal chromaffin granules. *Neuroscience* **5**, 1803–1823.