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MODULATION OF ACETYLCHOLINE-ELICITED CURRENTS IN CLONAL RAT PHAEOCHROMOCYTOMA (PC12) CELLS BY INTERNAL POLYPHOSPHATES

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SUMMARY

1. Whole-cell voltage clamp techniques were used to examine acetylcholine (ACh)-elicited currents in differentiated cells of the rat phaeochromocytoma cell line, PC12.

2. In the absence of intracellular Mg^{2+}, the whole-cell current–voltage relationship for the ACh-elicited current displayed inward rectification which was reduced in part by the presence of 5 mM internal adenosine 5′-triphosphate (ATP).

3. The reduction in the rectification attributed to ATP developed over the first 15–20 min of whole-cell recording. Similar results were obtained with a non-hydrolysable ATP analogue, adenosine-5′-*O*-3-thiotriphosphate (ATPγS), or cytosine 5′-triphosphate (CTP) in the internal solution, but not with adenosine 5′-diphosphate (ADP) or pyrophosphate.

4. The magnitude of the ACh-elicited current was also dependent on recording time and the composition of the internal pipette solution. The magnitude of the peak ACh-elicited current increased over time when the cell was internally perfused with the control solution or a pipette solution containing pyrophosphate, ATPγS, or ADP. The largest sustained increases in ACh-elicited current were observed in the presence of internal pyrophosphate or ATPγS. In contrast, with internal ATP or CTP, the whole-cell current initially increased, then steadily decreased with recording time.

5. The desensitization rate of the ACh-elicited current increased with recording time irrespective of the composition of the intracellular solution.

6. The actions of the compounds tested make it likely that the changes in the whole-cell current–voltage relationship, peak current, and desensitization are produced by separate mechanisms. The mechanisms underlying these changes are unknown, but the ability of the compounds to chelate divalent cations is unlikely to be the explanation. Other unlikely explanations include phosphorylation of the ACh receptor or regulation by GTP-binding proteins.

INTRODUCTION

Acetylcholine-elicited currents of the rat phaeochromocytoma cell line PC12 are inwardly rectifying, as are the ACh-elicited currents of various mammalian peripheral neurons (Derkach, Selyanko & Skok, 1983; Mathie, Cull-Candy & Colquhoun, 1987;

Yawo, 1989; Fieber & Adams, 1991), central neurons (Mulle & Changeux, 1990; Zhang & Feltz, 1990) and adrenal chromaffin cells (Hirano, Kidokoro & Ohmori, 1987; Maconochie, 1990; Inoue & Kuriyama, 1991). In PC12 cells, this rectification has been shown to depend on at least three voltage-dependent processes: the channel burst duration, single-channel conductance, and a fast process that closes channels at positive potentials (Ifune & Steinbach, 1990*a*, 1991, 1992). The burst duration of ACh receptors found on PC12 cells as well as those on rat sympathetic ganglion cells (Mathie *et al.* 1987; Mathie, Colquhoun & Cull-Candy, 1990) and rat submandibular ganglion cells (Yawo, 1989) are weakly dependent on voltage and although the dependence of the burst length on voltage does contribute to the whole-cell current rectification, it cannot fully account for the shape of the current–voltage (*I–V*) relationship.

The single-channel *I–V* relationship of nicotinic receptors found on rat sympathetic ganglion cells (Mathie *et al.* 1990) and PC12 cells (Neuhaus & Cachelin, 1990; Ifune & Steinbach, 1990*a*,) is dependent on intracellular and extracellular divalent cation concentrations and in the absence of divalent cations, the single-channel *I–V* relationship is linear. However, removal of divalent cations from the recording solutions does not remove the inward rectification of ACh-elicited whole-cell responses (Mathie *et al.* 1990; Neuhaus & Cachelin, 1990; Ifune & Steinbach, 1992). In PC12 cells, the remaining rectification can be accounted for by the voltage-dependent closing of nicotinic receptors at positive potentials (Ifune & Steinbach, 1992). In rat sympathetic ganglion cells, Mathie and his colleagues (1990) observed that the probability of being open for receptor channels in outside-out patches was greatly reduced at positive potentials immediately following excision.

Here, we show that the addition of ATP to the intracellular solution alleviates the rectification of the whole-cell current to the point where most of the voltage-dependence of the whole-cell conductance can be attributed to the voltage dependence of the channel burst duration. The effects of intracellular ATP take time to develop; 15–20 min of whole-cell perfusion is necessary before the effects are fully apparent. The effects of ATP and related compounds were examined in order to learn more about the mechanism underlying the relief of the current rectification. ATPγS and CTP were also able to reduce the rectification of whole-cell ACh-elicited currents, whereas in the presence of internal ADP or pyrophosphate, the rectification did not significantly differ from control. In addition to the whole-cell *I–V* relationship, two other characteristics of the whole-cell current changes with time; the magnitude of the whole-cell current and the rate at which the ACh-elicited current desensitized. In all cases, mechanisms responsible for the change are not apparent. However, the conditions used in the recordings make channel modulation by chelation of divalent ions, protein phosphorylation, or activation of GTP-binding proteins unlikely.

METHODS

Tissue culture. The PC12H cell line was kindly provided by Dr D. Schubert of the Salk Institute (San Diego, CA, USA). Cells were maintained and prepared for electrophysiological experiments as described previously (Ifune & Steinbach, 1990*b*). Cells were treated with 4 nM (100 ng/ml) mouse β-nerve growth factor (generously provided by Dr E. Johnson, Washington University School of Medicine, St Louis, MO, USA) and used 5–10 days after plating.

Physiological recordings. Current recordings were obtained using a List EPC-7 patch clamp

amplifier (List-Electronic, Eberstadt, Germany). Whole-cell records were acquired and stored using the PCLAMP programs (Axon Instruments, Foster City, CA, USA). Data were analysed using BINFITS (courtesy of Dr C. Lingle, Washington University School of Medicine) and DELP (courtesy of Dr D. Maconochie, Washington University School of Medicine). Patch pipettes were pulled from KG33 borosilicate glass capillaries using a P-80/PC micropipette puller (Sutter Instruments, San Rafael, CA, USA) and coated with Sylgard 182 (Dow Corning, Midland, MI, USA). After fire polishing, pipette resistances were 2–3 MΩ when filled with a low chloride intracellular solution. The series resistance during whole-cell recording ranged from 5 to 20 MΩ and was compensated 70–80%. All experiments were conducted at room temperature (23–24 °C).

Agonist-containing solution was perfused onto cells using a perfuser consisting of three glass capillary tubes (o.d. = 0·2 mm) glued together in parallel. The perfuser was placed approximately 50 μm away from the cell and between applications of agonist, the tube containing bath solution was directed towards the cell. To apply agonist, the perfuser was shifted so that one of the agonist-containing tubes was in front of the cell. The solution change around the cell was complete in approximately 50 ms. Solution was removed from the bath by a suction pipette placed at the edge of the recording dish.

Whole-cell current–voltage relationships were determined by measurement of peak currents elicited by 50 μM ACh at holding potentials between −80 and +80 mV. The peak inward current at −80 mV varied from cell to cell and also varied with recording time. Therefore, peak currents at all potentials were normalized to the peak current at −80 mV which was evaluated every 5–6 min during a recording. Currents were filtered at 2–3 kHz (−3 dB frequency) with an 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA, USA).

To quantify the changes seen in the desensitization rate with recording time, the decaying portion of the response to ACh was fitted with the sum of two exponential components. In the fitting the current was assumed to decay to baseline. The results of the fit were then used to calculate the time required for the current to decay to half its peak value.

Solutions. The extracellular solution contained (mM): 142 sodium isethionate, 8 NaCl, 1 $MgCl_2$, 20 Hepes, and 5 NaOH. The pH was 7·3 and the osmolarity, 320 mosmol/l. The extracellular bath solution also contained 500 nM tetrodotoxin to block voltage-gated Na^+ currents. The control intracellular solution consisted of (mM): 70 sodium isethionate, 10 NaCl, 20 EDTA, 40 Hepes, and 75 NaOH. For experiments in which the intracellular solution contained 5 mM ATP, adenosine-5′-*O*-3-thiotriphosphate (ATPγS), ADP, pyrophosphate, or CTP, the agent was added to a solution identical to the control intracellular solution except for the sodium isethionate concentration. In all cases, except for ATPγS, the sodium salt of the agent was used and the sodium isethionate concentration was adjusted to maintain a Na^+ concentration of 155 mM. ATPγS was used as a lithium salt and was added to the control solution without adjusting the Na^+ concentration.

The cell perfusion time constant (τ) was calculated using the formulas empirically derived by Pusch & Neher (1988) for bovine chromaffin cells. The following equation (derived from eqn (18) of Pusch & Neher, 1988) was used to calculate the time constants used in this study:

$$\tau = 0{\cdot}6(C/5{\cdot}91)^{1{\cdot}5}R_a M^{\frac{1}{3}}$$

where C is the capacitance of a PC12 cell (in pF), R_a is the access resistance in MΩ, and M is the molecular weight of the agent of interest in the pipette solution. The whole-cell capacitance and series resistance were measured using the transient compensation circuitry of the EPC-7. The molecular weights for the intracellular agents were as follows: ATP, 507; ADP, 427; CTP, 483; ATPγS, 518; and pyrophosphate, 174.

All drugs and chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA) unless specified otherwise.

RESULTS

Whole-cell I–V relationships

The current–voltage (*I–V*) relationship of PC12 cells in the absence of ACh was examined. Recording with a low intracellular Mg^{2+} and low chloride solution, the whole-cell *I–V* relationship was outwardly rectifying. Inward currents were relatively small and linearly increased as the holding potential was made more negative. At −80 mV, the leak inward current averaged 24 ± 13 pA (mean ± S.D.; $n = 42$). At

+80 mV, the steady-state outward current was 262±111 pA. An example of the whole-cell *I–V* relationship is given in Fig. 1 (○). The application of 50 μM ACh induced an inward current in PC12 cells held at −80 mV. At negative potentials, the inward ACh-elicited current increased in magnitude as the potential was made more

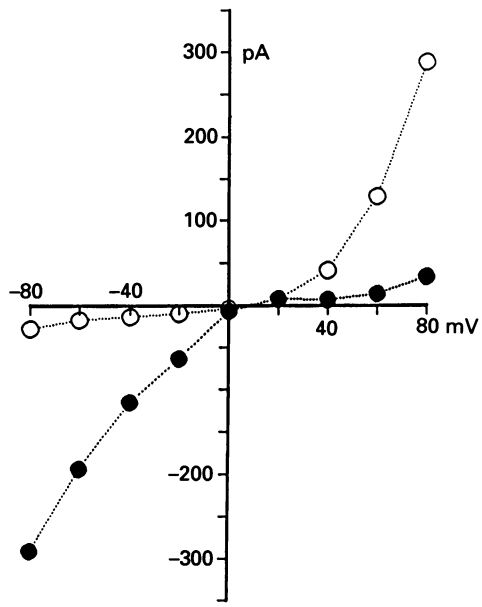

Fig. 1. *I–V* relationships for a PC12 cell in the presence and absence of ACh. The cell was held at various potentials between −80 and +80 mV. ○, steady-state whole-cell current in the absence of ACh plotted as a function of holding potential. ●, peak whole-cell current elicited by 50 μM ACh. The steady-state background current has been subtracted for this *I–V* curve. The intracellular solution contained nominally zero Mg^{2+} (20 mM EDTA, no added divalent cations).

negative. At positive potentials, the ACh-elicited current was outward in direction (the reversal potential was near 0 mV) and profoundly reduced in magnitude compared to inward currents. The current–voltage relationship for the ACh-elicited current is shown in Fig. 1 (●).

The inward rectification of the ACh-elicited current is a robust feature of the whole-cell current under the conditions described above. However, in the presence of intracellular ATP (5 mM), the *I–V* relationship of the ACh-elicited current displayed less rectification, an effect which developed with time after the establishment of the whole-cell configuration. Early in the recording, whole-cell ACh-elicited currents continued to display inward rectification (Fig. 2, right panel; ●). However, at later times (more than 20 min), the relative amount of outward current at positive potentials had increased (Fig. 2, right panel; ○). The *I–V* relationship in the negative voltage region was unaffected by ATP as was the *I–V* relationship of the cell in the absence of ACh. In the left panel of Fig. 2 are *I–V* curves, early (●) and late (○) in the recording, with control intracellular solution that did not contain ATP. In the absence of intracellular ATP, outward currents did increase slightly with time, but

not to the extent seen in the presence of intracellular ATP. Experiments were conducted to determine whether the alleviation of the rectification could be attributed to the ability of ATP to chelate divalent cations or if it could be due to a process such as receptor phosphorylation that requires the presence of ATP.

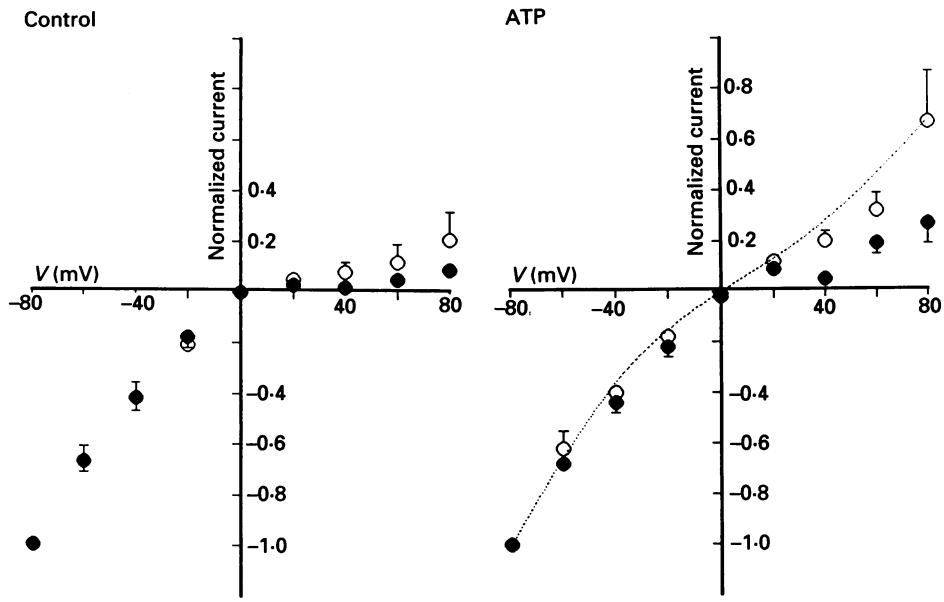

Fig. 2. Changes in the whole-cell *I–V* relationship of the ACh-elicited current with recording time. Presented are *I–V* curves determined during the first 10 min of recording (●) and late in the recording (○, ⩾ 15 min). Currents elicited by 50 μM ACh have been normalized to the peak current at −80 mV. The circles represent the mean (±S.D.) of the normalized whole-cell currents. Left panel, normalized *I–V* curves with the control intracellular solution ($n = 6$). Right panel, normalized *I–V* relationship with 5 mM intracellular Na_2ATP ($n = 5$). The dotted line represents the *I–V* relationship based on the voltage dependence of the probability of being open for single channels in outside-out patches (Ifune & Steinbach, 1992).

Effects of various intracellular agents on the whole-cell I–V relationship

To determine how ATP reduced the rectification of the whole-cell ACh-elicited current, we examined the effects of the following agents on the ACh-elicited current: ATP, ADP, ATPγS, CTP and pyrophosphate. The effects of these compounds on the whole-cell *I–V* relationship were analysed taking into account the effects of cell volume and series resistance on the rate at which compounds can diffuse into the cell from the pipette. A time constant for perfusion (τ) was estimated for each of the cells based on the relationship between the molecular weight of the compound under study, pipette access resistance, and cell capacitance (Pusch & Neher, 1988; see Methods). Values for the capacitance of PC12 cells ranged from 5 to 17 pF with a mean of 10 pF. The pipette access resistance ranged from 5 to 25 MΩ with a mean of 7·5 MΩ. Time constants ranged from 23 to 242 s. Of forty-eight cells, thirteen had a τ greater than 100 s; data from these cells were not used in the following analysis.

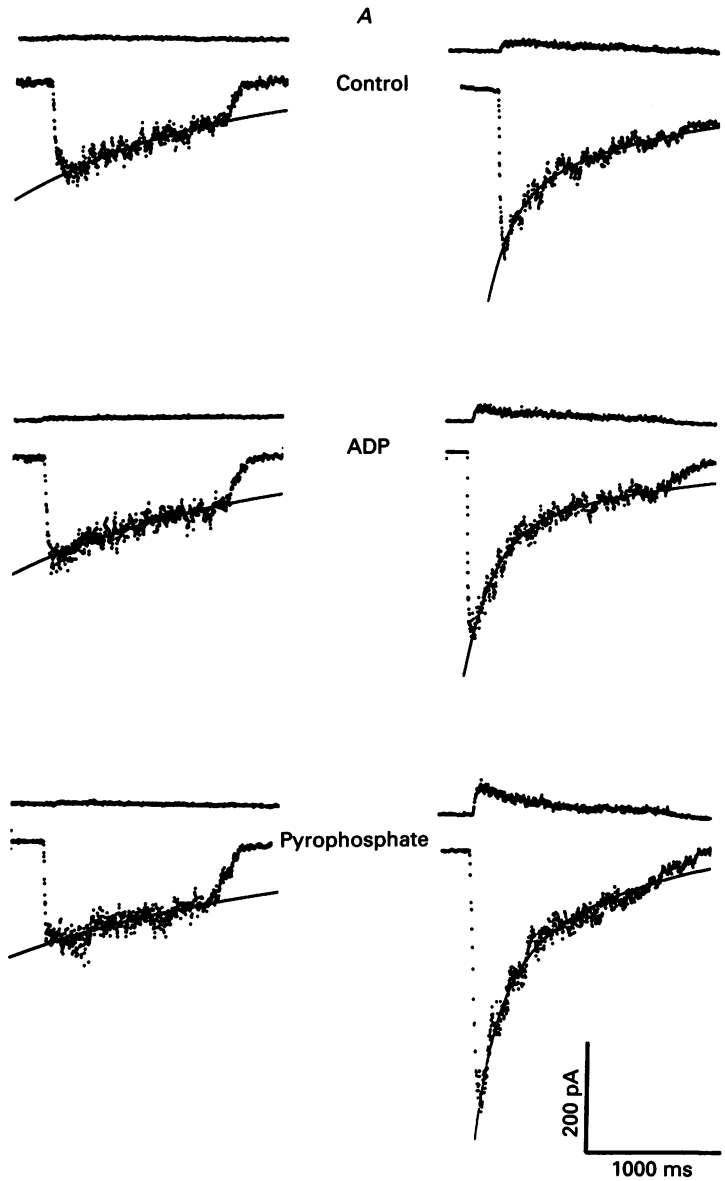

Fig. 3*A*. For legend see facing page.

All the time courses presented in this paper have been normalized using the perfusion time constants calculated for each cell.

In Fig. 3 are six sets of ACh-elicited currents from six different cells. Each cell was internally perfused with one of the intracellular solutions. There are two pairs of traces for each cell, each pair consisting of a trace at +40 mV and one at −80 mV. Pairs on the left were taken early in the recording period ($t < 4\,\tau$) and pairs on the right were recorded at a later time ($t > 20\,\tau$). In addition to ATP, two of the other compounds, ATPγS and CTP, were able to increase the relative amount of outward

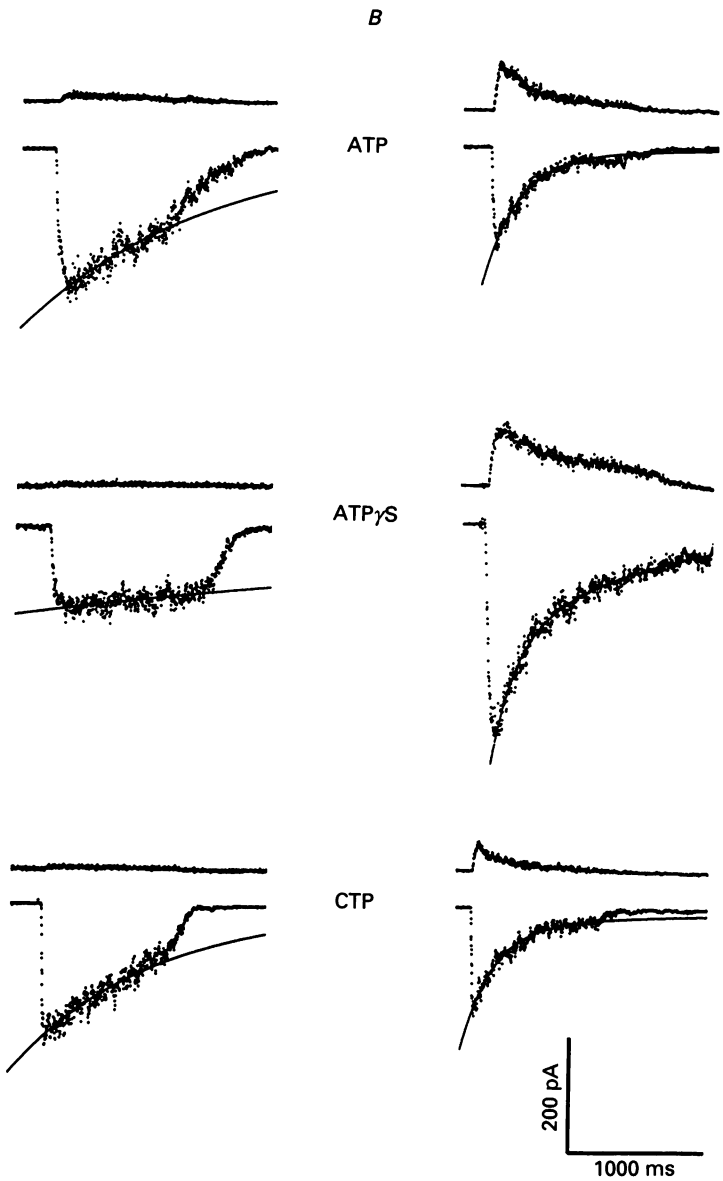

Fig. 3. Whole-cell currents at +40 mV and −80 mV elicited by 50 μM ACh with the various intracellular solutions. For each of the intracellular solutions, two pairs of traces from the same cell are shown. Each pair consists of a trace at +40 mV (upper trace) and −80 mV (lower trace). The pairs on the left were taken immediately after the initiation of the whole-cell recording and the pairs on the right were taken after at least 20 τ. The continuous line through the traces at −80 mV is a single-exponential curve fitted to the early traces and a double-exponential curve for the late traces. *A*, current traces from cells with the control intracellular solution (top), ADP (middle), and pyrophosphate (bottom). Values for $t_{\frac{1}{2}}$ with the control solution were 1·1 and 0·5 s at the early and late times, respectively; for ADP, 1·3 and 0·3 s; for pyrophosphate, 1·7 and 0·3 s. *B*, current traces from cells with intracellular ATP (top), ATPγS (middle), and CTP (bottom). Values for $t_{\frac{1}{2}}$ (early and late): ATP, 1·0 and 0·2 s; ATPγS, 3·7 and 0·4 s; CTP, 0·8 and 0·2 s.

TABLE 1. Comparison of rectification, magnitude and desensitization of ACh-elicited currents at the start and after 25 τ of internal perfusion

	$I(+40)/I(-80)$		$I(+40)$ (pA)	$I(+40)_t/I(+40)_i$	$I(-80)$ (pA)	$I(-80)_t/I(-80)_i$	Desensitization half-time (s)	
	at 2 τ	at 25 τ	at 2 τ	at 25 τ	at 2 τ	at 25 τ	at 2 τ	at 25 τ
ATPγS (3)	0·02±0·02	0·21±0·04	4·5±1·7	25·3±2·3	222±30	2·1±0·3	5·6±1·3	0·8±0·1
CTP (4)	0·03±0·03	0·20±0·02	11·9±8·2	24·4±13·4	338±59	1·2±0·2	3·4±0·7	0·5±0·1
ATP (5)	0·02±0·01	0·18±0·02	4·8±1·8	15·0±5·0	329±81	0·8±0·1	2·4±0·3	0·7±0·1
ADP (6)	0·02±0·01	0·12±0·02	3·5±1·1	13·8±2·4	230±39	1·4±0·3	3·3±0·3	1·0±0·1
Pyrophosphate (5)	0·01±0·01	0·12±0·01	2·8±0·6	24·6±6·5	206±26	2·1±0·2	4·6±0·8	0·7±0·1
Control (6)	0·01±0·01	0·06±0·02	3·1±0·8	9·8±4·4	271±46	1·5±0·2	2·5±0·4	0·9±0·1

The first column gives the compound added to the intracellular solution at a concentration of 5 mM. The number of cells in each group is given in parentheses. In columns 2 and 3 are the means of the ratio between the outward current at +40 mV and the inward current at −80 mV at 2 and 25 τ. If the whole-cell rectification is assumed to be entirely due to the voltage dependence of the channel burst duration, then $I(+40\text{ mV})/I(-80\text{ mV})$ is expected to be 0·2 (Ifune & Steinbach, 1992). Listed in column 4 are the means of the absolute peak current at +40 mV at 2 τ. In column 5 are the ratios between the peak current at +40 mV at 25 τ ($I(+40)_t$) and at 2 τ ($I(+40)_i$). Listed in column 6 are the means of the absolute peak current at −80 mV at 2 τ. In column 7 are the ratios between the peak current at −80 mV at 25 τ ($I(-80)_t$) and at 2 τ ($I(-80)_i$). In column 8 are the mean values for the $t_{\frac{1}{2}}$ of desensitization at −80 mV at 2 τ and in column 9 are values for the $t_{\frac{1}{2}}$ at 25 τ. All values are means ± S.E.M. Treatments are presented in order of decreasing effect on rectification (column 3). Note that enhancement of current (columns 5 and 7) and desensitization rates (columns 8 and 9) do not follow the same rank order of effect.

current to the point where the whole-cell *I–V* relationship depended solely on the voltage dependence of the channel burst duration. While normalized outward currents increased slightly with recording time in cells perfused with the control solution or solutions containing ADP or pyrophosphate, in each case the increase after 25 τ was not as great as that seen with cells perfused with ATP.

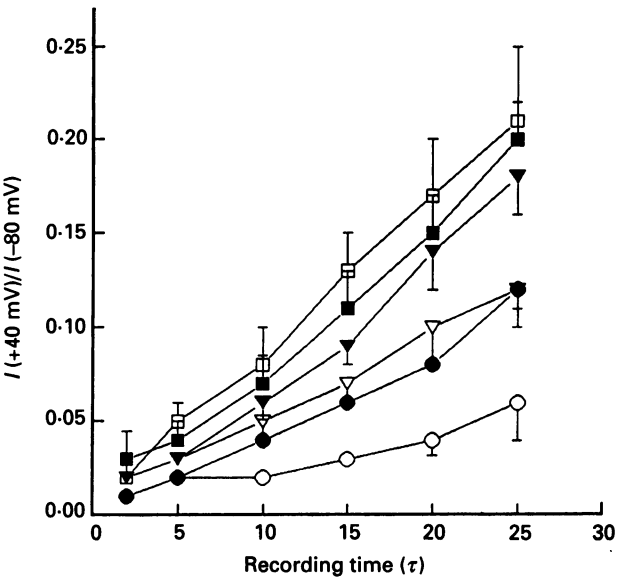

Fig. 4. Time course of the increase in the relative outward ACh-elicited current at +40 mV. The peak whole-cell current at +40 mV normalized by the current at −80 mV is plotted here as a function of time expressed as perfusion time constants (τ). Values are means (±S.E.M.) of the interpolated time points. ○, control ($n = 6$); ▼, ATP ($n = 5$); ▽, ADP ($n = 6$); ■, CTP ($n = 4$); □, ATPγS ($n = 3$); ●, pyrophosphate ($n = 5$).

In Table 1 are values for the mean of the normalized current at +40 mV at 2 and 25 τ. In the presence of intracellular ATP, CTP or ATPγS, the normalized outward current increased to levels significantly greater than with the control solution (Wilcoxon's rank test: $P < 0{\cdot}01$ for ATP ($n = 5$); $P < 0{\cdot}01$ for CTP ($n = 4$); $P < 0{\cdot}05$ for ATPγS ($n = 3$)). The normalized currents for cells perfused with ADP or pyrophosphate are intermediate at all times (see Fig. 4) and differ significantly at 25 τ only from those of cells perfused with CTP ($P < 0{\cdot}05$ for each). None of the agents that were added to the intracellular solution altered the characteristics of the *I–V* relationship of the background whole-cell currents (data not shown).

In Fig. 4, the normalized outward current is presented as a function of recording time. Each point represents the mean (±S.E.M.) of the ratio of the ACh-elicited current at +40 and −80 mV. Since the maximum inhibition of control current occurred at +40 mV, the relative amount of current at this potential provided a good indication of the degree of rectification that remained. In most cases, the ACh-elicited current at +40 mV was not evaluated exactly at 2, 5, 10, 15, 20 and 25 time constants after the start of the recording. Therefore, the ratio was calculated by linear interpolation of the existing time points.

In all cases, the relative outward current at +40 mV appeared to increase linearly with time. The rate at which the current increased was greatest when ATP, ATPγS or CTP was in the intracellular solution. With ADP and pyrophosphate, the rate of increase was intermediate and with the control solution, the rate was the slowest.

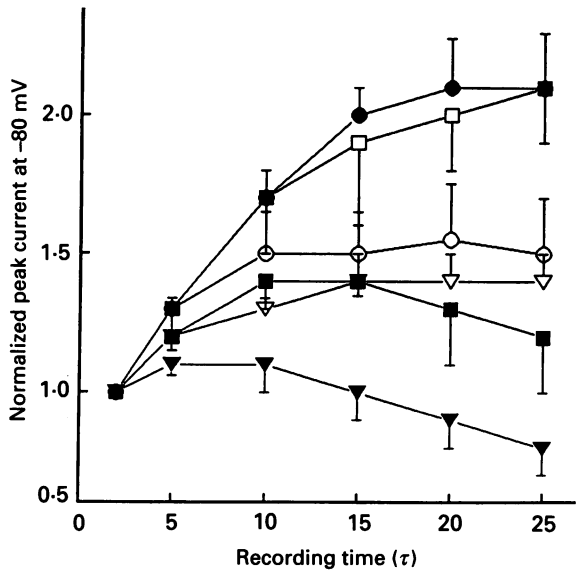

Fig. 5. Time course of the changes in peak current at −80 mV. The peak currents were estimated at 2, 5, 10, 15, 20 and 25 time constants (τ) after the start of the recording by linear interpolation of the existing data points. Each point represents the mean (±S.E.M.) of the peak current normalized by the peak current at 2 τ for cells internally perfused with control intracellular solution (○, $n = 6$) and solutions containing ATP (▼, $n = 5$), ADP (▽, $n = 6$), CTP (■, $n = 6$), ATPγS (□, $n = 3$), and pyrophosphate (●, $n = 5$).

Although this is not apparent in the plot, the relative outward current did not increase indefinitely. In cells with intracellular ATP and ATPγS for which the recording lasted longer than about 40 τ, the normalized current at +40 mV did plateau at 0·21 ± 0·02 ($n = 4$).

Changes in the ACh-elicited currents during whole-cell recording

In addition to the decrease in whole-cell rectification, there were two other characteristics of the whole-cell ACh-elicited current that changed with recording time: the magnitude of the current elicited by ACh and the rate at which the current desensitized. Examples of the change in the magnitude of the current with recording time can be seen in Fig. 3. When cells were perfused with the control solution or solutions containing ADP, pyrophosphate or ATPγS, the magnitude of the ACh-elicited current steadily increased with recording time. With ATP or CTP, the magnitude of the current initially increased, but at later times, decreased. For each of the intracellular agents, the increase in current at −80 mV is plotted as a function of normalized time in Fig. 5 and values are given in Table 1. The fraction of the initial current at −80 mV remaining at 25 τ for cells perfused with ATP differs significantly

from that for cells perfused with pyrophosphate, ADP or ATPγS ($P < 0.05$); and that for cells perfused with ADP also differs from the fraction of current remaining in cells perfused with either pyrophosphate or ATPγS ($P < 0.05$). Also shown in Table 1 are data for the ACh-elicited currents recorded initially at +40 mV, and the

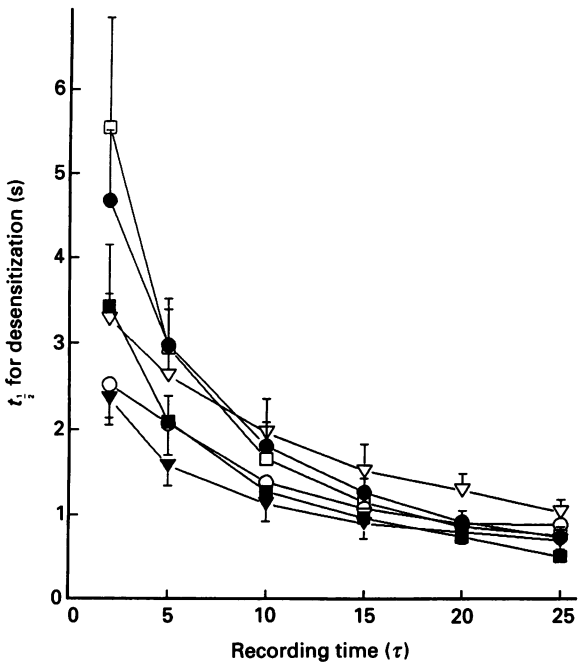

Fig. 6. Time course of the changes in desensitization. Each ACh-elicited current trace at −80 mV was fitted with one or the sum of two exponential components. The time required for the peak ACh-elicited current to decay by 50% ($t_{\frac{1}{2}}$) was estimated as described in Results. Since the current at −80 mV was usually not evaluated at 2, 5, 10, 15, 20 and 25 time constants, $t_{\frac{1}{2}}$ was determined by linear interpolation of the existing data points. Each point represents the mean ± S.E.M. for cells internally perfused with control intracellular solution (○, $n = 6$), solutions containing ATP (▼, $n = 5$), ADP (▽, $n = 6$), CTP (■, $n = 4$), ATPγS (□, $n = 3$), and pyrophosphate (●, $n = 5$).

relative current seen at 25 τ. For every cell, in every condition tested, current increased at +40 mV over the time course of the experiment. There were no significant differences in the relative increase in current elicited at +40 mV between the various treatments.

There was no consistent relationship between the increase in current at +40 and −80 mV between the groups. For example, with ATPγS, there was a large increase in current at both potentials. In contrast, with ATP, there was an increase in current at +40 mV and less current over time at −80 mV. Note, that in both of these cases, the whole-cell rectification was significantly reduced.

In addition to changes in the magnitude of the ACh-elicited current, the desensitization rate of the current increased with recording time. This was observed

with all the intracellular solutions (see Fig. 3) that were examined. To quantify the increase in desensitization rate, the falling phase of the current traces at −80 mV was fitted with one or the sum of two exponential components (see Methods). From this equation, the time required for the current to decay to half its peak value ($t_{\frac{1}{2}}$) was calculated. Examples of the fitted curves are shown in Fig. 3. At the earlier times, the desensitization could be described by a single-exponential equation. However, at the later times, two exponential components were needed to describe the currents well. In order to combine data from different cells, $t_{\frac{1}{2}}$ was calculated at 2, 5, 10, 15, 20 and 25 time constants after the start of the recording by linear interpolation of the time points. The time course of the increase in desensitization rate is shown in Fig. 6 for all the intracellular solutions. In Table 1, the mean $t_{\frac{1}{2}}$ values at 2 and 25 τ are given. Half-time values determined at 2 τ were significantly longer than those at 25 τ regardless of the composition of the intracellular solution (Wilcoxon rank test: control, $P < 0{\cdot}01$; pyrophosphate, $P < 0{\cdot}01$; CTP, $P < 0{\cdot}05$; ATP, $P < 0{\cdot}01$; ADP, $P < 0{\cdot}01$). The exception was with intracellular ATPγS, where although early $t_{\frac{1}{2}}$ values were consistently larger than those determined towards the end of the recording, the small sample size resulted in an insignificant difference. Cells that were internally perfused with ATPγS or pyrophosphate had a slower initial rate of desensitization compared to control cells. However, the difference was significant only for the comparison between the control and cells with internal pyrophosphate (Wilcoxon rank test, $P = 0{\cdot}05$).

Note also that desensitization does not depend strongly on membrane potential (see Fig. 3). It is likely that desensitization is somewhat slower at −80 than at +80 mV, because twenty-six of thirty-three paired comparisons of $t_{\frac{1}{2}}$ values showed larger values at −80 than +80 mV. However, the mean ratio was only about 1·3 (±0·2, mean±S.E.M.). If an exponential dependence of rate on membrane potential is assumed, this ratio would correspond to an e-fold change for more than 500 mV.

DISCUSSION

Three features of the ACh-elicited current changed with time of intracellular perfusion: rectification, absolute magnitude of the current, and rate of desensitization. The presence of intracellular ATP reduced the rectification of the whole-cell ACh-elicited current that remained in the absence of intracellular divalent cations. The alleviation progressively increased during the first 15–20 min of whole-cell recording. CTP and ATPγS had similar effects whereas ADP or pyrophosphate produced a lesser effect which was not significantly different to that seen with the control solution. The change in the magnitude of the peak current at −80 mV was also affected by the composition of the intracellular solution. With control solution or when the intracellular solution contained ADP, pyrophosphate, or ATPγS, the peak current at −80 mV increased with recording time. The largest increase was seen with pyrophosphate and ATPγS. With intracellular ATP and CTP, the peak current increased slightly then steadily decreased. The rate of desensitization and the ACh-elicited current at +40 mV increased over time with all internal solutions tested. From these results, one can conclude that the effects on rectification, current magnitude, and desensitization rate involve different mechanisms since the set of

compounds that affect the rectification are not the same as those that affect the other two processes (see Table 1).

The mechanisms underlying the changes in whole cell currents are not known. Two distinct (but not exclusive) possibilities are that the properties of already functional receptors change, or that additional functional receptors become apparent. PC12 cells express at least three conductance classes of receptor, the largest of which is most common and probably underlies most of the whole-cell current (Ifune & Steinbach, 1991). Previous work has indicated that the small initial relative current at +40 mV reflects the presence of a rapid channel closing process at positive potentials (Ifune & Steinbach, 1992). There is preliminary evidence to suggest that ATP reduces rectification by altering this rapid closing process. At early times during whole-cell perfusion the current relaxation at +40 mV after a jump from −80 mV comprised two exponentially decaying components; the larger amplitude component has a time constant of about 0·4 ms (Ifune & Steinbach, 1992). At later times in records from cells perfused with ATP the relative amplitude of the fast relaxation is reduced (C. K. Ifune & J. H. Steinbach, unpublished observations). In the case of ATP, the reduction of rectification is not associated with an increase in current at −80 mV, suggesting that only the rapid closing process is altered. In the case of ATPγS, the rectification is affected as much as by ATP but, in addition, the current at −80 mV doubles. Alternatively, in control solution the current at −80 mV increases with less change in rectification. The effects of control solution are more easily envisioned to reflect an increase in the number of functional receptors (cf. Margiotta, Berg & Dionne, 1987) with little change in the properties of the receptors. The action of ATPγS would reflect the superposition of these two independent effects – more functional receptors with altered properties. Of course, other mechanisms could also account for the observations, including a more complex modification of the voltage dependence of gating at both positive and negative potentials, or more complex regulation of the available numbers of two functionally distinct classes of receptors. The simplicity of two discrete and independent mechanisms – one determining the number of functional receptors and the other altering a single gating process – has some aesthetic appeal, but further work will be required to examine the actual basis for the effects seen.

The desensitization rate increases with time and the changes in rectification and in peak current amplitude may reflect altered desensitization. However, this possibility is made less likely by the following observations: (1) $t_{\frac{1}{2}}$ for desensitization is not strongly voltage dependent and (2) values for $t_{\frac{1}{2}}$ become the same at long internal perfusion times irrespective of the rectification or current amplitude. An increase in desensitization rate of ACh-elicited current with recording time has also been observed in adrenal chromaffin cells of the guinea-pig (Inoue & Kuriyama, 1991). Qualitatively, the phenomenon is similar to that seen in PC12 cells. However, Inoue & Kuriyama (1991) observed little change in the magnitude of the peak current with time.

There are many possible explanations for the effects that ATP has on the receptors. However, some of the more obvious possibilities appear unlikely. For example, the idea that ATP relieves rectification by acting as a more rapid chelator of divalent cations and preventing block of the receptor channels is not probable

since all the components used are fairly good chelators of divalent cations. The affinity of pyrophosphate for Mg^{2+} is comparable to that of ATP (Martell & Smith, 1974) and whereas ATP relieved rectification, pyrophosphate did not. Also, the length of time required to remove the rectification is very long compared to the amount of time required for the various agents to diffuse into the cell, while the Mg^{2+} activity should follow the local chelator concentration. That the rectification is not due to block of outward currents by Mg^{2+} or Ca^{2+} is consistent with observations made by other investigators. First, previous work on the ACh-elicited currents of neuronal cells had indicated that the rectification remained in the absence of intracellular divalent cations such as Ca^{2+} or Mg^{2+} (Hirano *et al.* 1987; Mathie *et al.* 1987; Yawo, 1989; Neuhaus & Cachelin, 1990; Ifune & Steinbach, 1992). Secondly, altering the reversal potential of the current does not change the voltage at which the maximum inhibition of current occurs (Yawo, 1990; Mathie, Cull-Candy & Colquhoun, 1991), which is inconsistent with a mechanism involving voltage-dependent block by divalent cations. These observations are more consistent with a process of rectification that involves a voltage-dependent conformation of the receptor channel. For these reasons, it is unlikely that the reduction in rectification results from the presence of a chelating agent inside the cell (in addition to the 20 mM EDTA present in all solutions).

A recent study by Sands & Barish (1992) has also examined the bases for rectification of ACh-elicited currents in PC12 cells, with very similar results to those presented here. These authors found that perfusion of cells with EDTA and ATP reduced the rectification along a time course similar to the one seen in the present study. They conclude, however, that the most likely explanation for their observations is the ability of ATP to chelate Mg^{2+} ions. The difference in interpretation probably arises from technical points. Sands & Barish (1992) report that the instantaneous whole cell I–V relationship is non-linear at early times after perfusion with EDTA plus ATP. Our experiments, however, had indicated that the instantaneous I–V relationship is linear at early times with this internal solution. We interpreted this to mean that Mg^{2+} block of the channel is rapidly relieved (Ifune & Steinbach, 1992) and that the whole-cell rectification reflects another rapid process which closes channels at positive potentials, with a time constant of about 0·4 ms. Voltage jumps during single channel openings (Ifune & Steinbach, 1990*a*) had indicated that block by internal Mg^{2+} ions, on the other hand, is essentially instantaneous. It is possible that the rapid closing process would not be resolved given the recording conditions (no series resistance compensation) and filtering (500–1000 Hz, 8-pole Bessel, low pass) employed by Sands & Barish (1992), and so the two 'instantaneous' I–V relationships actually would not be comparable. Hence, there is no essential difference in the data reported in the two studies. Our conclusion that the alleviation of rectification is not solely the result of divalent ion chelation is also influenced by the use of several putative Mg^{2+} chelators in addition to EDTA and ATP, and the very slow time course of the changes in comparison to the cell perfusion time constant.

Phosphorylation of receptor channels is also an unlikely explanation for any of the changes in the ACh-elicited current that were observed. CTP and ATPγS were equally as effective as ATP in reducing the rectification. This suggests that

hydrolysis of a high energy phosphate is not necessary for the removal of the rectification. Similarly, the agents that were seen to alter the magnitude of the peak ACh-elicited current and the rate of desensitization were not consistent with phosphorylation being the mechanism underlying these changes. Finally, the absence of Mg^{2+} (and therefore the absence of MgATP) in the intracellular solution would have made phosphorylation of the receptors an improbable event. In contrast, phosphorylation is thought to play a role in the modulation of whole-cell currents elicited by serotonin in NG-108 cells and ACh-elicited currents in chick ciliary ganglion cells. In NG-108 cells, the desensitization rate of the serotonin-elicited current becomes slower during whole-cell recording (Yakel, Shao & Jackson, 1991). Yakel and his colleagues suggest that this phenomenon is dependent on the hydrolysis of ATP because it is inhibited by the presence of non-hydrolysable ATP analogues in the internal solution. In chick ciliary ganglion cells, peak whole-cell currents in response to ACh are increased up to 3-fold in the presence of cyclic AMP analogues (Margiotta *et al.* 1987), which suggests a mechanism involving cyclic AMP-dependent protein phosphorylation (Margiotta *et al.* 1987).

The involvement of a GTP-binding protein is an explanation that would account for the long length of time required for the changes in the *I–V* relationship to reach completion. Although none of the intracellular solutions contained GTP, theoretically ATP, ATPγS and CTP could be converted into their guanosine counterparts to activate a G protein which would directly or indirectly affect ACh-elicited currents. Otero, Breitwieser & Szabo (1988), studying muscarinic potassium currents in bullfrog atrium, have reported that ATPγS can be converted into GTPγS by intracellular nucleoside diphosphate kinases during whole-cell recording. They observed that the delay in the activation of the muscarinic potassium currents was longer when the intracellular solution contained ATPγS than when it contained GTPγS. The difference in time was approximately 5 min (Otero *et al.* 1988). However, here, this explanation is unlikely for three reasons. First, there was no GDP in the pipette solution to act as a substrate for a putative nucleoside diphosphate kinase. Compared to atrial myocytes, PC12 cells are small and are unlikely to have the GDP reserves that appear to be present in the myocytes. Secondly, the outward current steadily increased with time beginning immediately after the whole-cell recording was established. Although it did take about 20 min for the process to reach completion, there was no initial delay before it started, which is what would be expected if some accumulation of GTP were needed before an effect is seen. Thirdly, there was nominally no free Mg^{2+} in the intracellular solution, which makes it unlikely that a G protein would be activated (Asano, Pedersen, Scott & Ross, 1984; Gilman, 1987). These arguments also reduce the likelihood of GTP-binding protein involvement in the changes seen in the magnitude of the ACh-elicited current and the desensitization rate of the current.

The loss or degradation of an intracellular factor may be the explanation for the changes in the magnitude of the peak current and the increase in the rate of desensitization. The time course of the changes in the whole-cell ACh-elicited current in PC12 cells is reminiscent of changes seen in other cells due to 'wash-out' of some intracellular factor(s). Waning of responses with whole-cell dialysis has been described for voltage-dependent Ca^{2+} currents in snail neurons (Byerly & Hagiwara,

1982; Chad & Eckert, 1986), serotonin response in hippocampal neurons (Yakel, Trussell & Jackson, 1988), and muscarinic response in rat lacrimal gland cells (Trautmann & Marty, 1984).

The effects of internal perfusion with the control solution are particularly surprising in comparison to the effects of patch excision (Ifune & Steinbach, 1991, 1992). In outside-out patches ACh-elicited channel activity shows no sign of the fast rectification process seen in whole-cell recordings, and hence rectification in the absence of Mg^{2+} ions is minimal. Further the activity rapidly runs down after excision. Hence, the rapid removal of most cytoplasmic components in identical internal solutions produce quite different effects in patches and perfused cells. This comparison suggests that the mechanism involved in modulating receptor function involves several factors, one or more of which are slowly diffusing, but which can be removed by excision.

In conclusion, three aspects of the whole-cell ACh-elicited current were observed to change with time: the whole-cell current–voltage relationship the magnitude of the peak current and the rate of desensitization. These changes occurred slowly with respect to the time course of internal perfusion. The presence of intracellular ATP altered the relative amount of outward current as well as having effects on the magnitude of the peak ACh-elicited currents. The effects of various intracellular agents were examined and the results suggested the following conclusions: (1) there are different mechanisms underlying each of the changes seen in the presence of internal ATP; (2) the changes do not require a high energy phosphate group; (3) protein phosphorylation is not involved; and (4) the changes do not require the activation of GTP-binding proteins. Although the physiological consequences of these changes are not known, the data suggest that such mechanisms may modulate the function of neuronal nicotinic receptors *in vivo*.

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